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Bacterial-Metal/Radionuclide Interaction: Basic Research and Bioremediation

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Bacterial-Metal/Radionuclide Interaction: Basis Research and Bioremediation

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Editors:

Sonja Selenska-Pobell, Heino Nitsche

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Invited and contributed oral presentations:

INVITED

AND

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SESSION I

ABSTRACTS

MICROBIAL DIVERSITY IN URANIUM MINE WASTE HEAPS

Axel Schippers, Regine Hallmann, Susanne Wentzien, Wolfgang Sand

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Two different uranium mine waste heaps near Ronneburg, Thuringia, Germany, which contain the remains of the activity of the former uranium-mining Soviet-East German company Wismut AG, were analysed for the occurrence of lithotrophic and chemoorganotropic leach bacteria. A total of 162 ore-samples were taken up to a depth of 5 m. Cell counts of iron(II) ion-, sulfur-, sulfur compound-, ammonia-, and nitrite-oxidizing bacteria were determined quantitatively by the most-probable-number technique. Sulfate-, nitrate-, iron(III) ion-, and manganese(IV) ion-reducing bacteria were also detected. In addition, the metabolic activity of sulfur- and iron-oxidizing bacteria was measured by microcalorimetry. Generally, all microorganisms mentioned above were detectable in the heaps. Aerobic and anaerobic microorganisms thrived up to a depth of 1.5 to 2 m. Up to 99 % of *Thiobacillus ferrooxidans* cells, the dominant leaching bacteria, occurred to this depth. Their numbers correlated with the microbial activity measurements and radon outgassing. Samples below 1.5 to 2 m exhibited reduced oxygen concentrations and reduced cell counts for all microorganisms.

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REMOVAL AND RECOVERY OF URANIUM BY USING MICROORGANISMS

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We have been working on the bioaccumulation of nuclear fuel elements, such as uranium and plutonium, by microorganisms from several angles. In our previous work, we have shown that some microorganisms, such as *Streptomyces albus* and *Chlorella regularis*, can accumulate large amounts of uranium from aqueous systems. Microbial biomass may thus be considered for use as an adsorbing agent for the recovery and removal of uranium that may be present in metallurgical effluents, mine tailings, and other waste sources.

The removal and recovery of uranium from aqueous systems by microorganisms has several advantages: (1) microbial cells have an extremely high uranium accumulating ability; (2) they can accumulate uranium very rapidly; (3) they can accumulate uranium selectively; (4) they produce microbial biomass inexpensively; (5) waste microbial cells by-produced in the amino acids fermentation industry can be used as an adsorbent for uranium; (6) both growing and immobilized cells of the microorganisms have the ability to accumulate uranium; (7) they pose no disposal problems.

In our previous screening tests concerning uranium uptake, extremely high uranium absorbing ability was found in *Pseudomonas stuzeri*, *Neurospora sitophila*, *Streptomyces albus* and *Streptomyces viridochromogenes*. In a uranium mine, it can be presumed that some microorganisms having a high accumulating ability for uranium, and different species of microorganisms having an ability to leach uranium from ore may exist in mine soil and aqueous systems. It would, therefore, be useful to find further microorganisms having an enhanced ability to accumulate uranium from uranium deposits.

Consequently, in this paper, hundreds of microorganisms existing in uranium mines located in Australia and North America, have been screened for their ability to accumulate large amounts of uranium. And we also discussed whether new strains of microorganisms found in various uranium deposits can be used for the bioprocessing of nuclear fuel elements.

Of these microorganisms tested, extremely high uranium accumulating ability was found in some strains appeared to be genus bacteria. One of them, *Bacillus subtilis* can accumulate about 615 mg of uranium per gram of microbial cells within 1 h. In North America we have also discovered some new strains, *Arthrobacter* sp. and *Bacillus subtilis*, having an extremely high ability to accumulate uranium. The uranium adsorbing capacity of these microbial cells exceeds two times that of commercially available chelating resins.

SOME FACTORS AFFECTING URANIUM ACCUMULATION BY MICROORGANISMS :

In order to obtain basic information on the bioprocessing of uranium by using new strains of microorganisms found in uranium deposits, some factors affecting uranium uptake were investigated in detail by using Bacillus subtilis isolated from Australian uranium deposits. The results obtained are as follows :

(1) Time course of the uranium accumulation

The amounts of uranium accumulated by the cells increase very rapidly during the first 3 minutes following the addition of uranium.

(2) Effect of pH on the accumulation of uranium

The amounts of uranium absorbed are highest at around pH 5 and decrease below pH 5. Thus, the accumulation of uranium by microbial cells is markedly affected by the pH of the solution.

(3) Effect of cell amounts on the accumulation of uranium

The amounts of uranium taken up by the cells (mg U / g microbial cells) decrease as the amounts of cells increase, whereas the total amounts of uranium taken up increase.

(4) Effect of external uranium concentration on the accumulation of uranium

The amounts of uranium absorbed by *Bacillus subtilis* (mg U / g microbial cells) increase as the external uranium concentration increases, whereas the ratio of the total amounts of uranium absorbed to the external uranium concentration decreases. When the external uranium concentration is low, the extremely high accumulation ratio is found. Bacillus cells can accumulate 615 mg uranium per gram of the microbial cells, and their uranium accumulating capacity exceeds two times that of commercially available chelating resins. The accumulation of uranium by the microbial cells obeys Langmuir isotherm.

On the basis of these facts, it seems reasonable to assume that the uptake of uranium onto microbial cells is almost dependent upon physico-chemical adsorption on the cell components.

SELECTIVE ACCUMULATION URANIUM BY MICROBIAL CELLS :

To determine which heavy metal ion can be most readily absorbed by microbial cells, we examined the selective accumulation of heavy metal ions by the cells from the solution containing 4×10^{-5} M of Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, and UO_2^{2+} at pH 5.

The relative order of magnitude of heavy metal ions absorbed by Bacillus cells appeared to be $UO_2^{2+} > Cu^{2+} >$ others. This result shows that *Bacillus subtilis* can readily accumulate uranium compared to other heavy metal ions.

ACCUMULATION OF THORIUM BY MICROORGANISMS :

The cells of *Bacillus subtilis* can also accumulate thorium as well as uranium with high efficiency. This strain can accumulate large amounts of thorium than uranium under the condition of pH 4.

RECOVERY OF URANIUM BY IMMOBILIZED MICROORGANISMS :

To obtain basic information on the recovery of uranium using the microbial cells immobilized with polyacrylamide, a repetition test of the uranium adsorption-desorption cycle was carried out.

The ability of the immobilized *Bacillus subtilis* cells to accumulate uranium did not decrease after 7 repetitions of the adsorption-desorption cycles in a column system. Thus, the immobilized microbial cells have excellent handling characteristics and can be used repeatedly in the adsorption-desorption cycles.

REMOVAL OF URANIUM FROM URANIUM REFINING WASTE WATER BY MICROORGANISMS :

We have tried to remove uranium from the uranium refining waste water by using some microbial cells having a high ability to accumulate uranium. These microbial cells can remove uranium from the uranium refining waste water with high efficiency.

CONCLUSION :

In uranium deposits located in Australia and North America, we have discovered some new microorganisms having an extremely high ability to accumulate uranium. These strains appeared to be genus bacteria can accumulate about 615 mg of uranium per gram of the microbial cells, and their uranium accumulating capacities exceed two times that of commercially available chelating resins.

Accordingly, we consider that these new microorganisms can be used as an adsorbing agent for the removal of the nuclear fuel elements which may be present in metallurgical effluents, mine tailings and other waste sources.

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MOLECULAR CHARACTERIZATION OF *THIOBACILLUS* STRAINS RECOVERED FROM A URANIUM MINING WASTE PILE

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Bioleaching involves solubilization of metals from minerals by metabolic activty of mixed microbial populations. *Thiobacillus ferrooxidans* is the most frequently used bacterium for commercial leaching of metals such as copper, uranium, and gold from sulphide containing ores. Thiobacilli as well as many other acidophilic, chemoautotrophic and heterotrophic bacteria have been found in uranium mines/1/. *Thiobacillus* strains recovered from a former uranium mine in Saxony, Germany, were characterized using pulsed- field gel electrophoresis (PFGE) /2/ and repetitive primer amplified polymorphic DNA (rep-APD) fingerprinting /3/.
These two methods derive information from the whole bacterial genome. In the case of PFGE, the embedded bacterial cells were lysed and the intact genome DNA was digested by the use of a rare cutting endonuclease *XbaI*. The obtained DNA fragments were separated in an alternating electrical field. Genomic fingerprinting by rep-APD is based on the use of special oligonucleotide primers complementary to interspersed bacterial repetitive sequences and PCR. As a result differently sized DNA fragments consisting of sequences laying between these elements were obtained. The latter were fractionated by electrophoresis /4/.
Using the above mentioned methods six newly isolated *Thiobacillus*-isolates were characterized. As shown in Fig. 1, the PFGE fingerprints of the waste isolates were sample-specific. The sampling sites had different depths and metal compositions. Three of the isolates, TFSS1, TFSS2, and TFSS6 were recovered from a sample drawn from a depth about one meter under the surface, where the concentration of uranium was low. The three other samples TFSS3, TFSS4, TFSS5 are recovered from a depth between two and three meters. The concentration of uranium at this site was estimated to be three times higher than those in the other sample mentioned above.

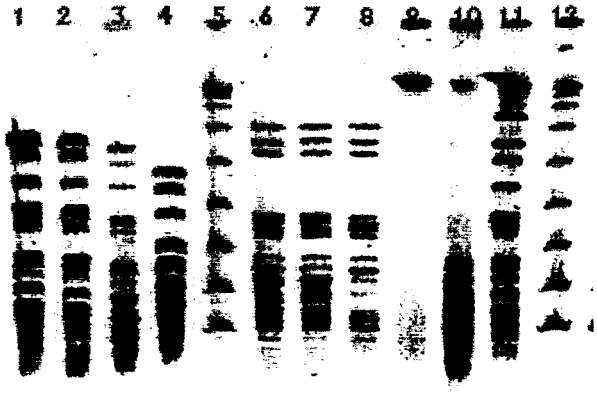

Fig. 1: PFGE- Fingerprint of the strains: 1,*Thiobacillus ferrooxidans* ATCC 19859; 2,*Thiobacillus ferrooxidans* ATCC 21834; 3,*Thiobacillus ferrooxidans* ATCC 23270; 4,*Thiobacillus ferrooxidans* ATCC 33020; 5,λ Ladder PFG Marker; 6,Pile isolate TFSS1; 7,Pile isolate TFSS2; 8,Pile isolate TFSS6; 9,Pile isolate TFSS3; 10,Pile isolate TFSS4; 11,Pile isolate TFSS5; 12,λ Ladder PFG Marker

The six novel *Thiobacillus ferrooxidans* isolates form a particular rep-APD group which is closely related to the strain *Thiobacillus ferrooxidans* ATCC 33020, recovered from an Uranium mine in Japan (see Fig. 2).

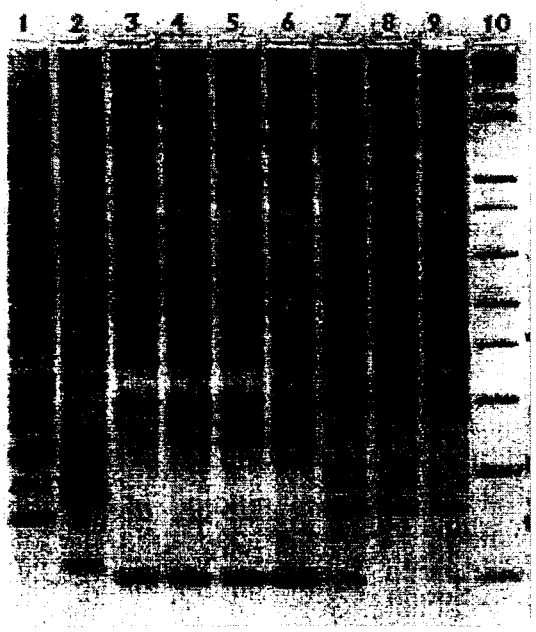

Fig. 2: Genomic rep-APD fingerprint of the strains: 1,E. coli; 2,Thiomonas cuprina DSM 5495; 3,Pile isolate TFSS6; 4,Pile isolate TFSS2; 5,Pile isolate TFSS1; 6,*Thiobacillus ferrooxidans* ATCC 33020; 7,*Thiobacillus ferrooxidans* ATCC 23270; 8,*Thiobacillus ferrooxidans* ATCC 21834; 9,*Thiobacillus ferrooxidans* ATCC19859;
10, 1kB Plus DNA Ladder

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SULPHATE REDUCING BACTERIA AND THE SEASON VARIATION OF TECHNETIUM UPTAKE RATE BY FRESHWATER LAKE SEDIMENTS

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Investigation of radionuclides' accumulation by natural associations of microorganisms contained in the sediments is important for comprehension of their behaviour in ecosystems. It can also provide us with the biomethod for contaminated water treatment. The absorption of the long-lived 99Tc (VII) by the samples of the fresh-water silt, taken from the eutrophic lake Beloye, Kosino (Moscow region) and a typical dystrophic lake (Shatura region). The kinetics' analysis has shown that the half-time values of the initial Tc content removal from the eutrophic lake water phase were about 350 - 450h. Hydrosulfide, produced by sulfate reducing bacteria, that are the component of the silt, is thought to be responsible for the Tc precipitation.

Some difference in Tc bioaccumulation rate by the silts, taken in winter (from under the ice cover) and in summer periods from the eutrophic lake Beloye, especially in the case of sterilized samples, was found out. However the temperature dependence of Tc uptake rate was not very high, the uptake halftimes increasing from 16 to only 18 days with the temperature decrease from 15°C to 6 °C. This was partly associated with the hydrosulfide concentration decrease in winter period due to the artificial aeration and the depression of metabolism at low temperatures. At the same time it was shown that the hydrosulfide concentration is not the only parameter being important for the intensity of Tc uptake. The influence of concentration and diffusion factors as well as the presence of humic acids in the samples taken from dystrophic lake were also studied.

The grey silt samples from Lake Beloye were shown to be effective sorbents for technetium for the periods of more than 2 years for the solutions containing up to 20 mg/l of Tc. Feeding with cut reed could prolong and increase the decontamination activity. Application of natural association containing sulphate-reducing bacteria for contaminated ponds treatment is discussed.

MOLECULAR ANALYSIS OF NATURAL BACTERIAL COMMUNITIES

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Molecular biological methods have provided powerful new ways to study microbial communities in natural environments. Phylogenetic analysis of 16S ribosomal RNA gene sequences, an approach pioneered by Woese and his associates [1,2], can be used to determine how microbes cultured from natural environments are related to one another and to previously described microbial taxa. This approach yields information on the identity, diversity, and possible functions of the isolated strains, along with data that can be useful in the development of more direct molecular methods. The direct methods can be used to study natural microbial communities "directly" in environmental samples, without having to culture the microbes therein. Examples of this approach include the use of 16S rRNA-based probes to detect specific types or groups of microbes microscopically [3] and phylogenetic analysis of 16S rRNA gene sequences cloned from nucleic acids extracted directly from environmental samples [4, 5]. Direct molecular methods allow one to study the unculturable members of a community that are not detected by culture-based approaches. To obtain the most complete picture of diversity in natural microbial communities, however, it may be necessary to use a combination of direct molecular and culture-based methods, at least until the direct approaches are more fully developed [5].

The research described in this paper focused on phylogenetic analysis of 16S rRNA gene sequences to characterize bacteria cultured from natural communities, specifically those in terrestrial subsurface environments (environments situated beneath the topsoil zone of the Earth's crust). The bacteria were cultured from saturated and unsaturated environments that were situated 50 to 2,700 m below land surface and were composed mostly of various types of unconsolidated materials (sands, gravels, clays, paleosols, etc.). The isolates were obtained by researchers associated with the U. S. Department of Energy's Deep Microbiology Program and were preserved in the Subsurface Microbial Culture Collection (SMCC) at Florida State University. The phylogenetic analyses are part of a long-term effort to better characterize and organize the bacteria in the SMCC.

Phylogenetic analysis of 16S rRNA gene sequences has shown that the subsurface strains in the SMCC represent a wide range of diversity. To date, these strains have been placed in six major phylogenetic/taxonomic categories: the high- and low-G+C Gram-positive bacteria; the alpha-, beta-, and gamma-subdivisions of the *Proteobacteria*, and the *Flexibacter/Cytophaga/Bacteroides* group [6]. Within these groups, the isolates have been assigned to at least 40 distinct genera, although 72% of them fell within just 9 genera. The most frequently encountered Gram-negative genera were *Comamonas, Acinetobacter, Caulobacter, Pseudomonas,* and *Sphingomonas*; the most often seen Gram-positive genera were *Arthrobacter, Streptomyces, Staphylococcus,* and *Bacillus.* Within groups of isolates falling into a single genus, consider-

able diversity at the species and strain levels was detected by 16S rRNA sequence analysis and various DNA fingerprinting methods.

Phylogenetic analyses have also indicated that novel organisms have been isolated from subsurface environments. About 5% of the isolates examined phylogenetically are not closely related to any genus in the current 16S rRNA sequence data bases and, thus, could represent novel genera. Most of the subsurface strains clearly fall within established genera, but many of them appear or already have been shown to be novel species within those genera. Among the better characterized novel strains are three new species of *Sphingomonas* that can degrade a variety of aromatic and polyaromatic compounds [7].

Phylogenetic analyses have detected differences in the composition of the culturable bacterial communities in different types of subsurface environments. For example, the communities in saturated paleosols, lacustrine sediments, and fluvial gravels at a site in Washington State were considerably more diverse (in terms of the number of genera isolated) than those in saturated Atlantic coastal plain sediments (mostly sands) at a site in South Carolina. For the most part, different genera were detected at the two sites, although two genera were among the most frequently isolated forms at both sites [6]. The bacterial communities in vadose (unsaturated) zone samples from sites in Washington State differed markedly from those in saturated subsurface environments in that they consisted almost entirely of Gram-positive forms, most of which were spore-formers (such as *Bacillus* and *Streptomyces*) that were seldom seen in samples from saturated environments.

In the process of characterizing isolates in the SMCC, several strains were detected that are of importance in regard to metals and radionuclides. A vadose-zone isolate, ZAN-044 (probably a strain of *Bacillus simplex*) was found to biosorb cobalt, nickel, cadmium, and strontium. A strain of *Shewanella* from deep saturated sandstones at a site in New Mexico reduces Fe(III) and U(VI), and a rather unusual new anaerobic species of *Bacillus* from 2,700-m-deep shales at a site in Virginia reduces Fe(III) and Mn(IV). A new species *Thermus* from deep South African gold mine reduces Fe(III) complexed with citrate or nitrilotriacetic acid, as well as Mn(IV), Co(III), EDTA, and Cr(VI). Screening of the SMCC to look for additional strains that interact with metals or radionuclides is now under way. Several more Fe(III)- and/or U(VI)-reducing isolates have been detected thus far, and these are currently being characterized.

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MICROBIAL LIFE IN DEEP GRANITIC ROCK

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Our research on microbial life in deep granitic rock aquifers [1] has been performed at seven sites at depths down to 1500 m: The Stripa research mine in the middle of Sweden, the Äspö hard rock laboratory (HRL) situated on the south eastern coast of Sweden and on four sites in Finland. One of the goals with this research is to understand how subterranean microorganisms may interact with the performance of a future repository for high level nuclear fuel waste (HLW) [2]. Therefore, work has been performed in co-operation with other national and international research groups at additional sites in Sweden, Canada and at the natural nuclear waste analogue sites Oklo in Gabon, Palmottu in Finland and Maqarin in Jordan. The ultimate limitation for an active microbial life at depth is suggested to be the availability of hydrogen, and possibly methane, as energy sources over time and these gases have indeed been found in most deep groundwater. The finding of a deep, autotrophic hydrogen-based biosphere [3] adds a significant but previously overlooked reducing activity in the deep rock aquifers. Sulphate and iron-reducing bacteria use organic material from the autotrophs and expel sulphide and ferrous iron. A good correlation has been shown between viable iron and sulphate reducing bacteria and the amount of ferric iron and sulphide precipitates in groundwater conducting aquifers. Different microbially catalysed reactions that reduce oxygen in accordance with available nutrients have been demonstrated in the deep granitic Äspö HRL environment. A HLW repository will contain oxygen at start, which may be corrosive for waste canisters and promote incidental radionuclide migration. The observed microbial oxygen reducing activity will rapidly reduce this risk. Recent data from Äspö HRL indicates that in addition to organisms representing the domains Bacteria and Archaea, organisms from the domain Eukarya, yeast and fungi, are also present and probably intrinsic. The influence from complexing agents produced by bacteria, yeast and fungi on radionuclide migration remains to be studied at realistic conditions in deep groundwater.

Altogether, our results show that there is a very high probability for the existence of an intra-terrestrial biosphere that is driven by hydrogen from the interior of the earth and, therefore, independent of photosynthesis. Our planet obviously has two biospheres, the sun driven biosphere that is well known and accepted by everybody, and the new, unexplored earth driven intra-terrestrial biosphere. Prospective research will aim at exploration of distribution, diversity, *in situ* activity and biogeochemistry of the intra-terrestrial biosphere and its influence on anthropogenic underground operations.

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URANIUM ADSORPTION BY BACILLUS SP.: IMPLICATIONS FOR RADIONUCLIDE TRANSPORT THROUGH SOILS

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The ability of bacteria to bind radionuclides is well documented. In addition, the potential of microorganisms to nucleate mineral precipitation, accelerating the formation of inorganic compounds such as amorphous ferric oxyhydroxides, is being increasingly recognised. In a subsurface environment, where microbial cells frequently constitute one of the dominant organic components of the solid phase matrix, bacterial surfaces may therefore play an important role in the transport and attenuation of actinide elements, such as uranium.

Uranium, used in this study as a model contaminant, exhibits a complex aqueous chemistry, reflected in the formation of multiple oxyanions and carbonate species. Previous work has illustrated a notable affinity of this element for biosorption. This study has examined the uranium binding capacity of *Bacillus subtilis* 168. Preliminary results have shown that uranium is directly sorbed onto the surfaces of untreated bacterial cells, with equilibrium being established in less than forty minutes. TEM micrographs illustrated substantial uranium uptake by both intact and lysed bacterial cells. TEM/EDS analysis also identified secondary removal of uranium via coprecipitation with and/or adsorption to cell associated ferric oxyhydroxide precipitates. Preliminary results appeared to suggest that uranium removal to iron coated cells may be more efficient than direct sorption to untreated cells.

Further studies will compare the uranium sorption potential of abiotically generated amorphous iron oxyhydroxides with those that are biogenically formed. Differences in the nature and capacity of uranium binding by viable and non-viable cells will also be assessed. In addition, uranium sorption by *Bacillus subtilis* 168 will be compared quantitatively to that of other *Bacillus* sp.

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SCANNING SURFACES FOR SPECIFIC BACTERIAL ENZYME ACTIVITY AND MOLECULAR IDENTIFICATION AND ISOLATION OF THE MAIN BACTERIA

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The identification of the predominant bacteria in the environment is strongly hampered by the fact that most native bacterial cells are hardly accessible for cultivation methods. This problem can be bypassed by direct isolation of bacterial nucleic acids from the environment and analysis of the 16S rRNA sequence, allowing a phylogenetic affiliation without real cells on hand. However, this approach is dependent on the efficiency of nucleic acids extraction and the reproducibility of sampling. The minute amounts of DNA or rRNA needed to analyse microbial communities can be obtained from small environmental samples but are often meant to represent a large environmental matrix. Aquatic samples can be representative by filtering large volumes of water and using the residue on the filter for nucleic acids extractions. Soil samples are more difficult, since only a few gram are used for nucleic acids extractions. Here, pooling and mixing of the samples is useful to yield a mean [1]. Dry surfaces, providing a 'two-dimensional' environmental matrix with normally minute biomass amounts to sample, are not to analyse in this way. The patchwork-structure of the microbial community, for instance on rocks [2], demands for covering more or less the whole investigated surface. However, sampling the whole surface would severely damage if not destroy the microbial community. A non-invasive scanning method and spatially selective sampling is required. We adapted a method of scanning rock surfaces with 4-methylumbelliferyl-bound substrates and combined it with bacterial isolation and 16S rDNA analysis by temperature gradient gel electrophoresis. Firstly, the selected area of a rock surface (500 cm^2) is covered for one hour with a flexibly supported agarose gel containing the 4-methylumbelliferyl-bound substrate. Specific enzyme activities, as present in the active microbes, release 4-methylumbelliferone. Then the gel is incubated on an alkaline wet filter, where 4-methylumbelliferone develops strong fluorescence under UV-light. In this way, specific microbial enzyme activities can be located on the rock surface. Further, the agarose gel traps some loose particles from the surface like airborne dust-sediments from rock niches and also bacterial cells. Samples can be taken from the enlightened spots on the gel and be used for a selective cultivation approach with the investigated substrate. Now the widely undisturbed rock surface can be selectively sampled for DNA extraction. The generation of community fingerprints via PCR and TGGE allows to search for the really predominant rock organisms among the cultured isolates. With this approach the distress to analyse surface communities turns to a fortune: Now it is possible to link structure and function of environmental communities - a goal which actually is hardly achievable for soil and water samples. This method will be useful in microbial ecology to identify microbial communities on rocks, buildings and artwork or in biotechnology to study colonization and microbial activities in solid phase bio-reactors.

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INVITED

AND

CONTRIBUTED ORAL

PRESENTATIONS

SESSION II

ABSTRACTS

BIOLEACHING: MOBILIZATION AND RECOVERY OF METALS FROM SOLID WASTE MATERIALS

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A series of metabolic reactions can be induced when microbes are exposed to metals: (i) soluble metals can be transported through the cell membrane by ion pumps, ion channels, or other carrier compounds and are accumulated within the cells (bioaccumulation); (ii) metals can be extracellularly bound on cell surfaces (biosorption); (iii) metals can be reduced or oxidized resulting in changes of the metal mobility; (iv) certain metals can be alkylated resulting in an increased metal mobility (volatilization); (v) the microbial secretion of acids can result in changes of the metal mobility; (vi) the microbial formation of complexing or chelating agents can lead to an increase or decrease of metal mobility.

The main mechanisms regarding the mobilization (leaching) of metals from solids are acidolysis, redoxolysis, and complexolysis. However, it is suggested that a combination of all three mechanisms might be responsible for metal solubilization. Under appropriate environmental conditions, metals are solubilized and extracted from metal-rich materials in subsurface ecosystems by the action of bacteria and fungi. As a consequence, microbial metal leaching represents a potential environmental hazard in mine tailings or landfills. However, these microbial activities can be applied in the industry for the recovery of metals from solid materials. The technology has successfully found practical application in copper and gold mining ("bioleaching", "biomining") where low-grade ores are biologically treated to obtain metal values which are not accessible by conventional (mechanical or thermal) treatments [1]. Metals can be obtained from leachates by suited techniques (e.g. precipitation, cementation, or electrowinning). Besides industrial interests, the application of bioleaching technologies must also be seen in the context of a sustainable future in which industrial technologies have to be increasingly in harmony with the global material cycles of the biosphere.

A wide variety of both lithotrophic and organotrophic microorganisms is known to mediate the mobilization of various elements from solids mostly by the formation of inorganic (e.g. sulfuric acid) or organic acids (e.g. citric gluconic, lactic, tartric, malic, or oxalic acid) as well as for the ability to catalyze redox processes [2]. In general, autotrophic organisms (CO_2 as carbon source) are forming inorganic acids whereas heterotrophic organisms (organic carbon sources) are forming organic acids. In ore treatment facilities mainly autotrophic *Thiobacillus* and *Leptospirillum* species have been identified as mediators of solubilization reactions.

Several processes have been established in industrial applications for a microbial metal mobilization such as *in situ*, dump, heap, or tank leaching [3, 4]. For heap leaching, low-grade ores are treated on a water-impermeable foundation by a continuous irrigation in a closed system with aqueous media containing microorganisms and certain nutrients. The leachate is collected for further processing. Tank leaching is the technically most sophisticated method and allows a

process control regarding stirring, pH, temperature, or aeration. A series of patents on bioleaching processes for metal winning from solids including besides ores also fly ash, sewage sludge, and other metal-containing industrial wastes has been granted [1].

Besides technical and ecological advantages, bioleaching methods have also some economical benefits: leaching efficiencies are increased in comparison to physico-chemical methods (as demonstrated by processing gold-containing ores); energy input and maintenance costs are low. Bioleaching can compete with conventional (thermal) techniques. Since its development the proportion of biologically recovered metals has increased steadily. Today, approximatively 20% of the gold and 30% of the world copper production is based on biological methods resulting in an added value (according to market prices of raw materials) of over 10 billion US$.

In current research projects, microbiological leaching processes have been applied for the mobilization of metals from different metal-containing solid wastes as well as metal-contaminated soil (Tab. 1). Due to high contents of certain metals these waste materials can be considered as "artificial ores".

Tab. 1. Selection of metal-containing solids treated by a biololeaching process (b.d.: below detection limit; n.d.: not determined.

Material	Source	Metal content (g/kg)							
		Al	Cd	Cr	Cu	Ni	Pb	Sn	Zn
Fly ash	Municipal waste incineration	70	0.5	0.6	1	0.1	8	8	31
Bottom ash	Municipal waste incineration	46	b.d.	0.4	1.5	0.1	0.7	0.3	2.5
Dust	Electronic scrap	240	0.3	0.7	80	15	20	20	25
Sludge	Galvanic industries	n.d.	n.d.	26	43	105	n.d.	n.d.	166
Soil	Mining activities	n.d.	0.1	0.2	9	0.1	25	1.6	24

Pure cultures of bacteria (*Thiobacillus ferrooxidans, T. thiooxidans, Pseudomonas putida, Bacillus megaterium*) [5] and fungi (*Aspergillus niger, Penicillium simplicissimum*) [6] as well as mixed cultures and enrichments (e.g. from sewage sludge) have been evaluated for their potential to leach metals from solid wastes. Growing these organisms in the presence of fly ash from municipal waste incineration, it was possible to solubilize e.g. Cd, Cu, or Zn to an extent of >90 %, whereas for Cr, or Ni leaching efficiencies of only 10 to 20 % were determined. The comparison of different microbial strains suggested a selectivity for certain elements (Fig. 1). *Aspergillus niger* was able to leach upto 60% of Pb. In contrast, when *Thiobacillus thiooxidans* was grown on ash, only 3% of Pb was solubilized. The results show the potential of different microorganisms to leach substantial quantities of metals from fly ash. Depending on the point of view, the mobilization or bioleaching of these metals could be considered either as a hazard for the environment (leachates from landfills or deposits) or as a possibility to reduce the content of toxic elements to fulfill regulations for landfilling or for a re-use of fly ash (e.g. for construction purposes).

On a pre-industrial scale a bioleaching process has bee developed to treat copper and iron containing sulfidic sludges from electroplating industries. Upto now in Switzerland, such materials have to be stored in safe but extremely expensive repositories or they have to be exported with high costs (about US$ 850 per ton). The leaching circuit consisted of a bioreactor unit, an extraction stage, and two washing steps. Sulfidic sludge (70 - 200 g Cu kg^{-1} dry wt) is oxidized with

ferric iron in a $2m^3$ reactor, followed by filtration. The copper-bearing leaching solution was afterwards subjected to electrowinning, where copper was recovered as pure metal. The residual ferrous iron was oxidized to ferric iron by *Thiobacillus ferroxidans* and the solution was recycled and used as lixiviant in the leaching stage. A process flowsheet and the material balance showed the chemical and biological feasibility of the leaching circuit. Valuable metals can be recovered and the costs of disposing galvanic sludge are drastically reduced, because the residues can be deposited on a lower cost level in landfills instead of expensive repositories.

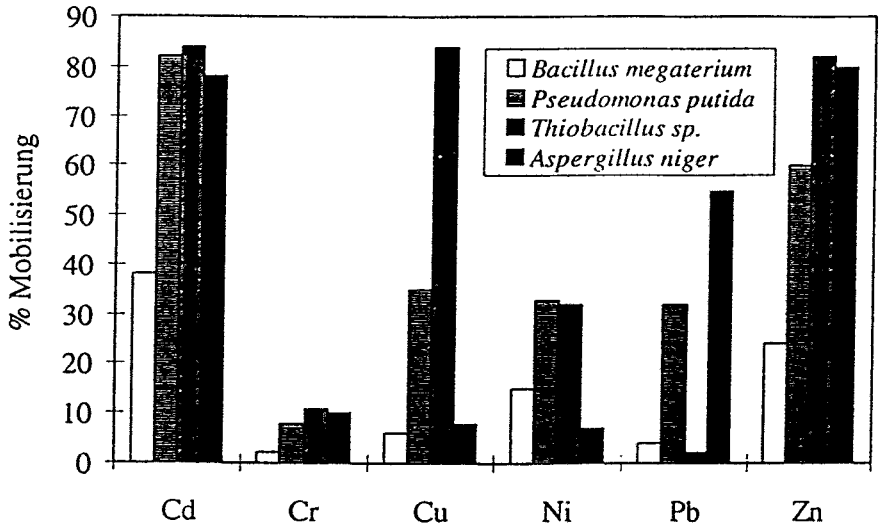

Fig. 1. Metal mobilization from municipal waste incineration fly ash in a 2% suspension by different microorganisms such as *Bacillus megaterium, Pseudomonas putida, Thiobacillus* sp., and *Aspergillus niger.*

In conclusion, microbial treatment of metal-containing waste materials would contribute to environmental protection efforts in different areas: (i) stimulation and realization of the "cycling concept" regarding wastes; (ii) protection of resources such as land (landfills), energy, and ore deposits; (iii) availability of new resources such as "artifical ores" produced during industrial processes. In addition, bioleaching techniques offer also possibilities for the bioremediation of metal containing soils.

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BIOLEACHING OF HEAVY METAL CONTAMINATED SOILS

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Heavy metal-contaminated soils represent a serious hazard to the environment, and other than with organic contaminants they cannot be decomposed biologically, chemically or physically. However, heavy metals may be solubilized, transported into the groundwater and may be accumulated and incorporated into plants, animals and man. Currently it is possible to decontaminate only to a certain extent; therefore contaminated soils and residues must normally be disposed of in a special landfill. Even there heavy metals, contaminating the seepage water,may be dissolved by bio-leaching processes due to microbial acid production, e.g. formation of sulphuric acid by oxidation of sulphur and reduced sulphur compounds, by the excretion of organic acids, by changes in oxidation states ($U^{IV+} \rightarrow$ to U^{VI+} ; $Cr^{3+} \rightarrow Cr^{6+}$), and by microbial oxidation of ferrous iron to ferric iron which then functions as a chemical oxidant.

Based on these mechanisms biotechnological methods have been developed for the mobilization of heavy metals from ores and mineral industrial waste products [1]. Only a few efforts have been made for applying this technique for remediation of contaminated soils [2], because the main leaching organisms (*Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans*) have been found to be sensitive to even low concentrations of organic substances that are often present in soils [3].

In a preliminary study soil samples from different contaminated sites (Table) were investigated for the presence of metal-mobilizing bacteria. Enrichment of microorganisms was performed with four different media for growing sulphur-, thiosulphate- or Fe^{2+}- oxidizing bacteria. Metal-mobilizing bacteria were found in all of the soil samples by at least one type of medium. The enrichment cultures solubilized heavy metals to varying extents, depending on the enrichment medium, the soil sample, and the type of contaminant. More often cadmium and zinc were totally extracted form the soil, whereas copper and nickel were solubilzed up to a

certain degree. Arsenic extraction was favoured by sulphuric acid producing bacteria and up to 80 % As was solubilized from a tannery sampling site. Lead was not observed to have been mobilized at all. In some cases the heavy-metal contamination was reduced to such an extent that threshold values were reached recommended for almost unrestricted use of the soil. The results of these studies are promising for the success of work on developing a biotechnological process for detoxifying soils contaminated with heavy metals.

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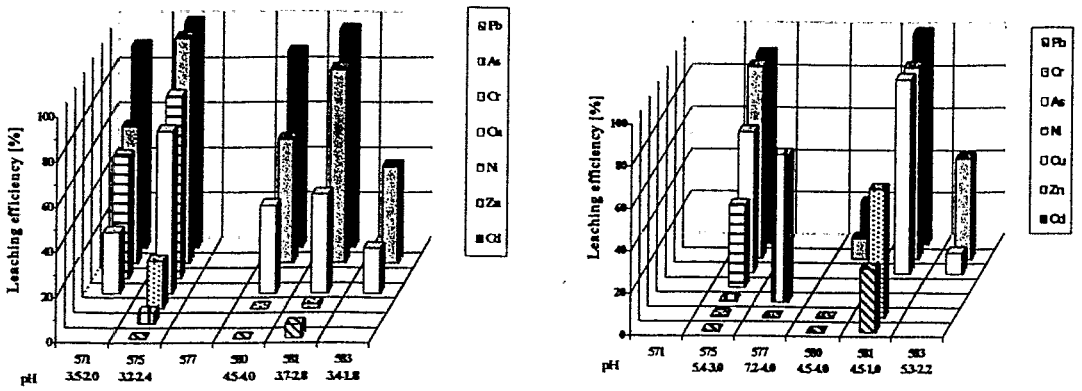
Table: Concentrations of contaminants (mg/kg) and pH in heavy metal contaminated soils. Concentrations exceeding German threshold values (LAGA) are shown in bold letters.

Sampling site	number	pH *	As	Cd	Cr	Cu	Ni	Pb	Zn
galvanic plant	571	7.8	17	**277**	58	**351**	**2612**	85	**1891**
rainwater basin sludge	572	6.3	9	**3**	38	**112**	57	98	225
scrap yard	575	7.3	**276**	**20**	**228**	**2296**	**104**	**19138**	**2865**
goat´s leather tannery	577	8.3	**4115**	2	**1176**	46	11	104	72
hand-grenade test site	580	6.3	11	**76**	**111**	**2423**	52	200	**1612**
hand-grenade test site	581	5.6	22	**80**	**158**	**1349**	58	**321**	**1889**
shipyard's slipway	583	7.7	9	<1	53	**175**	5	145	**497**
LAGA threshold		5-9	50	3	100	100	100	200	300

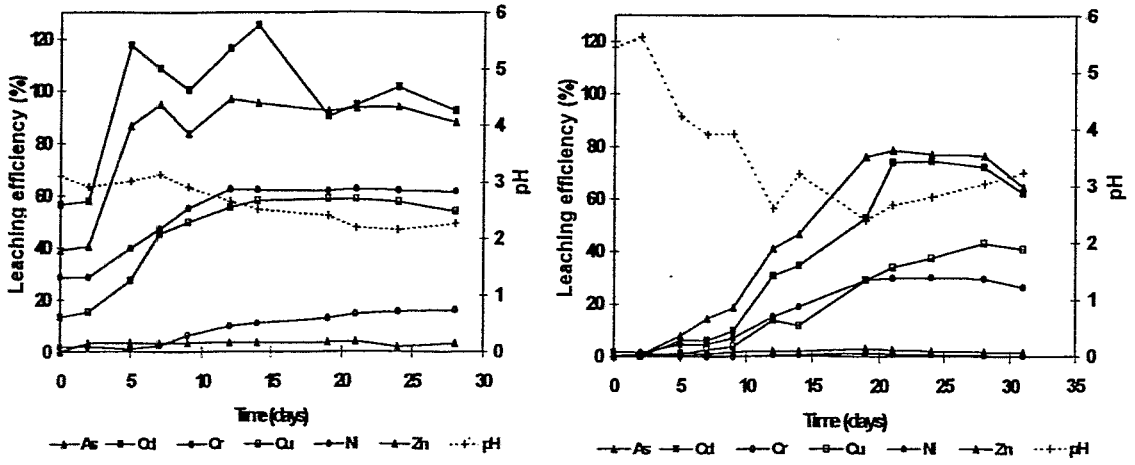
* = pH of 25 ml of water with 10 g dried sample after one hour of agitation.



Iron oxidizing bacteria (M)

Sulphur oxidizing bacteria (S)

Fig.1. Metal extraction (%) by iron and sulphur oxidizing enrichment cultures



Iron oxidizing bacteria (M)

Sulphur oxidizing bacteria (S)

Fig.2. Kinetics of bacterial leaching of sample 575 with iron oxidizing enrichment cultures in Mackintosh medium (M) and sulphur oxidizing enrichment cultures in Starkey medium (S). Leaching efficiencies of lead were less than 0.5 % and are not shown.

THE IMPORTANCE OF CHEMISTRY IN METAL - MICROORGANISM INTERACTIONS

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ABSTRACT

The investigation of metals, including radionuclides, interactions with microorganisms has intensified in recent years as biological solutions to environmental pollution are seen as opportunities for microbiologists to demonstrate the potential of this technology. This eagerness to transfer environmental biotechnology to commercial reality is partly due to the successful clean-up of major oil spills, as with the Exxon Valdez, using microorganisms.The use of microorganisms to treat land, silts, ground water contaminated with organic pollutants is now well established. These microbial methods can compete in many cases on efficiency and economics. This however is not the case with the treatment of effluents, including land, contaminated with toxic heavy metals and/or radionuclides.There are no known examples of microorganisms being used at commercial or even demonstration scale to remediate toxic metal contaminated land. Of the four pilot plant studies investigating the potential of bacteria to remove metals from various liquid wastes undertaken in the last fifteen years only one has been commercialised. Why so few pilot plant studies and the poor transfer rate to the market place?
This paper will address the metals-microorganism interactions from the chemist perspective. The chemical behaviour of metals, including radionuclides, will be described with particular attention to

the influence of environmental conditions such as pH and Eh on their speciation.The coordination of the various ligands associated with both dead and living cell systems either at the cell wall or intercellular will be compared with their synthetic counterparts with particular attention to the thermodynamic and stereochemistry considerations.
In many cases, their is a lack of fundamental chemical knowledge, this is particularly true for the actinides even with simple ligands such as sulphide. This paucity of technical information and its impact on the future development of environmental biotechnology will be addressed.

MICROBIAL REDUCTION OF URANIUM AND OTHER METAL CONTAMINANTS BY DISSIMILATORY Fe(III)-REDUCING MICROORGANISMS

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Microorganisms with the ability to conserve energy to support growth via the oxidation of organic matter or hydrogen coupled to Fe(III) reduction, are found in phylogenetically diverse groups throughout the *Bacteria* and the *Archaea*. In pristine environments these organisms, known as dissimilatory Fe(III)-reducing microorganisms, primarily use Fe(III) and Mn(IV) as terminal electron acceptors. However, many of these Fe(III) reducers also have the ability to reduce a variety of other metals and metalloids and this metabolism has potential applications for the remediation of metal contamination.

For example, most dissimilatory Fe(III)-reducing microorganisms can reduce U(VI) to U(IV). This is significant because U(VI) is soluble, and thus mobile, in most subsurface environments whereas U(IV) precipitates in most natural waters and waste streams. Studies with a variety of Fe(III) reducers have demonstrated that they can rapidly reduce U(VI) and precipitate U(IV) as the insoluble mineral, uraninite. Microbial uranium reduction has been shown to effectively precipitate uranium from a number of contaminated waters including groundwaters from a Department of Energy site and surface waters from a uranium mining site. Furthermore, U(VI) reducers can be used as part of a extraction and precipitation strategy to concentrate uranium from contaminated soils.

Dissimilatory Fe(III) reducers can also reduce Co(III) complexed with EDTA to Co(II). This can promote immobilization of radioactive cobalt in the subsurface because Co(II) is not strongly complexed by EDTA and tends to adsorb to particulates. These organisms can also reduce soluble Tc(VII) to insoluble Tc(IV) and Tc(V). Reduction of Np(V) to Np(IV) has been demonstrated, with the Np(IV) precipitated using inorganic phosphate liberated via the phosphatase of *Citrobacter* sp. Plutonium may also be reduced. Some Fe(III) reducers can reduce soluble toxic Cr(VI) to the less soluble, less toxic Cr(III), and soluble Hg(II) can be reduced to volatile Hg°. The soluble metalloid

contaminant Se(VI) can be reduced to insoluble Se°. As(VI) can be reduced to As(III) which, depending upon conditions, can result in solubilization or precipitation of arsenic.

Fe(III) reducers might also be helpful in extracting a variety of trace metals from contaminated soils. This is because many trace metals are strongly sorbed onto Fe(III) oxides in soils. When Fe(III)-reducing microorganisms reduce these highly insoluble Fe(III) oxides, the trace metals are **no** longer tightly bound and may be easier to extract.

Fe(III) reducers must be in direct contact with metals in order to reduce them. However, in the presence of humic acids, the requirement for direct contact is alleviated because the Fe(III) reducers can transfer electrons to humics and the microbially reduced humics can then nonenzymatically reduce the metals. Metals that can be reduced via this electron shuttling mechanism include not only Fe(III) and Mn(IV), but also contaminants such as Cr(VI), Hg(II), U(VI), and Tc(VII). Quinone moieties are the primary electron-accepting group that is involved in electron shuttling. Organics other than humics which also contain quinone groups can also serve as electron shuttles. The addition of humics or other extracellular quinones can greatly accelerate the rate of metal reduction. It can also permit the reduction of metals that otherwise would not be accessible for microbial reduction when the metals are occluded in pore spaces too small for microorganisms to enter.

Molecular analysis of field and laboratory studies in which metal reduction in aquifers has been stimulated with the addition of electron donors and/or humics or humics analogs have demonstrated that bacteria in the genera *Geobacter* generally become the predominant metal-reducing microorganisms. This illustrates that it is important to understand the mechanisms of metal reduction in *Geobacter* species. Studies with *Geobacter sulfurreducens* demonstrated that this organism contains a membrane-bound NADH-dependent Fe(III) reductase comprised of a NADH dehydrogenase, ATPase subunits that might be involved in metal binding, and a *c*-type cytochrome which appears to be the Fe(III) reductase. Current research is focused on developing molecular analyses which can quantify the activity of the reductase in subsurface environments in order to monitor the effectiveness of in situ remediation of metal-contaminated subsurface environments.

SELECTIVE BIOSORPTION OF METALS BY BACTERIA: FACTORS OF INFLUENCE

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Numerous studies in recent years have shown that microorganisms can take up considerable amounts of heavy metals, or radionuclides and may play an important role in the concentration and migration of these elements under natural conditions. One of the interaction processes is biosorption. This mechanism can be considered as the first step in the microorganism-metal interaction. It encompasses the uptake of metals by the whole biomass (living or dead) through physico-chemical phenomena such as adsorption, ion exchange or microprecipitation. These processe take place on the cell wall surface and they have fast kinetics [1]: the uptake equilibrium state is obtained in a few minutes.

This presentation described some of the factors affecting the biosorption capacity and the selectivity of a microbial biomass.

The first factor of influence on the biosorption capacity is the physiologic state of the bacteria. We have observed that *Pseudomonas aeruginosa* adsorbe more uranium when the cells are harvested in the stationary phase of growth. For *Mycobacterium smegmatis*, if the cells are starved for 15 days, the biosorption capacity of gadolinium is higer (Table 1).

Furthermore, the pH of the medium has a significant effect on biosorption, caused by his influence on the metal speciation and on the global charge of the cell wall. The latter is generally negative at neutral pH. In solutions of high ionic strenght we observed a charge reversion of the cell layers. Then it is possible to fix anionic species at basic pH.

The diversity in the nature and in the reactivity of the metallic ions leads to variations in the amounts of metal uptake. In the case of the lanthanide ions, it could be observed a certain selectivity in the capacity of fixation by *Pseudomonas aeruginosa* [5].

Another factor influencing the capacity of the microbial cell wall to "catch" the metal ions is the nature and the compostion of this layer. The diversity in the wall structures leads to variations in the fixation capacity. In the table 1, we present the maximum capacity of biosorption of gadolinium (Gd) for different species of microorganisms.

The binding capacity is greatly affected by the growth medium and by the environmental conditions. To illustrate this fact, cells of *Alcaligenes eutrophus* CH 34 have been harvested from "rich" and "poor" medium. These two biomasses don't have the same capacity of Gd adsorption (Table 1), the rich medium shows better biosorption. The determination of adsorption isotherms has lead to the following results. The "poor growth cell" presents an adsorption isotherm of the Langmuir type (single layer adsorption) and the "rich growth cell" obeys to the Brunauer-Emmett-Teller (BET) model (multi-layer adsorption).

The presence of soluble exopolymers in the medium could affect the biosorption capacity. The fixation of nickel by activated sludges is disrupted by the presence of

soluble biopolymers [6] and the fixation of uranium species on chitosan is inhibited by the presence of humic substances [7].

Microorganism	Cell wall structure	Element	Biosorption µmol.g-1 (dry biomass)	References
Mycobacterium smegmatis	Acido-alcolo resistant	UO_2^{2+}	187	2
		Th^{4+}	170	2
		La^{3+}	24	3
		Eu^{3+}	126	3
		Gd^{3+}	110	This work
		Gd^{3+} (a)	190	This work
Bacillus subtillis	Gram +	Gd^{3+}	350	Thiswork
Pseudomonas aeruginosa	Gram-	Gd^{3+}	322	4
		La^{3+}	397	5
		Eu^{3+}	290	5
		Yb^{3+}	326	5
Alcaligenes eutrophus CH 34	Gram-	Gd^{3+}	147	4
		Gd^{3+} (b)	40	4
Saccharomyces cerevisiae	Glycan	Gd^{3+}	7	4

(a) cell who are starve 15 days in stationary phase.

(b) cell growth in a poor medium

Table 1: Maximum capacity of biosorption in function of some microorganism species and metal ions (error ±10%).In the case of Gd all experiments were carried out at pH 5 and with 4 % wet biomass.

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SELECTIVE BIOSORPTION OF LANTHANIDE (La, Eu, Yb) IONS BY *PSEUDOMONAS AERUGINOSA*

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The removal of metallic ions La^{3+}, Eu^{3+} and Yb^{3+} from aqueous solution by adsorption on *Pseudomonas aeruginosa* biomass was investigated. The lanthanide biosorption equilibrium obeyed the Brunauer-Emmett-Teller isotherm model, indicating multi-layer adsorption. Bacterial cells showed high affinities at low concentrations of lanthanide cations under our experimental conditions (see [1, 2]). Maximum adsorption capacities were found: 397 $\mu mol.g^{-1}$ for lanthanum, 290 $\mu mol.g^{-1}$ for europium and 326 $\mu mol.g^{-1}$ for ytterbium (± 10%). The results indicated that there were about 100 specific sites for lanthanum per g of dry biomass. The diversity of potential metal-binding groups was revealed by potentiometric titrations of the biomass, giving some information concerning the amount of strong and weak acidic functional groups (0.24 ± 0.05 $mequiv.g^{-1}$ and 0.86 ± 0.02 $mequiv.g^{-1}$, respectively). Experiments with mixed-cation solutions showed that the sequence of preferential biosorption was: $Eu^{3+}=Yb^{3+}>La^{3+}$. The uptake of lanthanide by *P. aeruginosa* cells was not affected by the presence of sodium, potassium, calcium, chloride, sulfate and nitrate ions. Aluminium was a strong inhibitor of lanthanide ions biosorption. 87% of the total Al^{3+} was removed from the 3 mM solution, whereas only 8%, 20% and 3% of the total La^{3+}, Eu^{3+} and Yb^{3+}, respectively, were sorbed from 3 mM solutions. The results suggested that cells of *Pseudomonas aeruginosa* may find promising applications as inexpensive biosorbent materials for removal and separation of lanthanide ions from aqueous effluents.

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METAL CLUSTER DEPOSITION ON BACTERIAL SURFACE LAYER PROTEINS

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The deposition of nano-size metal clusters on membranes is of high interest for possible chemical and physical application. In the paper it will be shown that bacterial surface layer proteins (S-layer) can be used successfully for manufacturing of such functionalized membranes. S-layer sheets were isolated from *Sporosarcina ureae* and from *Bacillus sphaericus*. The lattice constant of the 2D protein crystal equals 13.2 nm , and 12.5 nm, respectively. The metal was deposited via liquid phase [1]. Pt clusters were formed by reduction of K_2PtCl_4 complexes, whereas for the Pd cluster deposition the S-layer protein solution was treated with a Na_2PdCl_4 solution. The structure of the activation products has been investigated by TEM. The composition of adsorbed metals clusters has been determined by EDX-analysis. The deposition of platinum or palladium clusters from the aqueous solution is governed by selective adsorption in the pore channels of the protein surface. The formed metal clusters are characterised by a very small size distribution and a closed packed, quasi periodic distribution at the S-layer [2]. By means of additional marker experiments with polycationic ferritin the dominating mechanism for pattern formation has been explained as a competition between charge compensation and site specific interaction.

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Biosorption and Bioreduction of Heavy Metals by *Arthrobacter sp.* BP 7/26

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Arthrobacter sp. BP 7/26 is able to biosorb up to 280 mg of silver per gram dry weight. The binding occurs passively within 30 minutes, without the participation of metabolism, and is completely reversible if nitric acid is used as the desorbent. The involvement of extractable cell wall components, e.g. of proteins, glycolipids or fatty acids, in biosorption of silver was relative low (not more than 20%). However, reactive chemical groups in the murein-sacculus of *Arthrobacter sp.* BP /26 were the most important binding sites. With chemical modification of electropositive amine groups in the cell wall of this strain the enrichment of silver, copper and nickel significantly decreased. The chemical masking of electronegative carboxyl groups caused a drop in biosorption of copper, nickel and palladium of more than 50%.
Strain BP 7/26 produces high amounts of metabolites during growth, in at least 240 mg of silver per hour is reduced to metallic Ag^0 form. This immobilisation was proved by means of diffraction analysis to represent a biocrystallization of the metal, which seems not to be dependent on a silver resistance of the bacterial strain.
Transmission-electron-microscopy was used to examine morphological details, which complemented and extended the chemical analysis, and localised the silver also within the cell, where a regular distribution and several globular aggregates of the metal were observed. As binding sites within the cells, storage compounds (e.g. glycogen), amino acids and deoxyribonucleic-acid may be of relevance. There are indications that all these cell-components offer nucleation points for the crystallization of silver as well.

INVITED

AND

CONTRIBUTED ORAL

PRESENTATIONS

SESSION III

ABSTRACTS

ROLE OF BACTERIA FOR DECONTAMINATION OF NUCLEAR FUEL CYCLE WASTES

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The nuclear fuel cycle produces, in addition to the intensely radioactive high-level wastes from the nuclear fission process, large volumes of low-level waste comprising uranium, fission products and transuranic elements in aqueous solution, dilute nitric acid or, in the special case of decontamination solutions, chelating agents. Extractive processes and nuclear fuel reprocessing can generate waste tributyl phosphate (TBP) containing residual metals [1].

For treatment of fission products standard physico-chemical and biotechnological processes are applicable. ^{99}Tc (as TcO_4^-) is problematic but this can be remediated via a biotransformation described elsewhere in this volume (J.R. Lloyd *et al*). Bioremediative measures not relying on simple biosorption commonly utilize bioprecipitation using biogenic (e.g.) sulfide or phosphate ligands. Sulfate-reducing bacteria convert SO_4^{2-} to H_2S with concomitant removal of metal sulfides, a process particularly applicable to, for example, uranium mine waters, where sulfate is present to excess. For other wastes where nitrate ion is to excess new strains of denitrifying bacteria may have potential (C. Sahut, pers. comm.).

Biogenic phosphate precipitation (by an atypical *Citrobacter* sp., *Escherichia coli* containing the appropriate cloned gene *phoN* or by *Acinetobacter* sp.) relies on the hydrolysis of organic phosphate or polyphosphate species; the inorganic phosphate product effluxing from the cell intercepts incoming metal to form cell-bound polycrystalline metal phosphate species [2,3]. The trivalent and hexavalent transuranic elements (e.g. Am(III) and U(VI) and Pu(VI)) are removed as, respectively, $AnPO_4$ and $HAnO_2PO_4$ (An = actinide element). However the ease with which the transuranic elements can change valence, and thus acquire a new set of chemical properties, presents problems. The tetravalent species (e.g. Th(IV), Pu(IV), Np(IV)) can be remediated by such precipitative methods but with low efficiency; this is attributed to the solution chemistry of the actinide ion in a given ionic matrix. The pentavalent Np(V) species, the most common form in solution (as NpO_2^+) is very mobile in the

environment, does not form tight metal-ligand complexes and, accordingly, is very difficult to remove by established technologies.

Initial studies using a chemical 'surrogate' system established that pre-deposition of the phosphate of an innocuous metal ($LaPO_4$) created a nucleating surface upon which the deposition of metal phosphate of the more 'difficult' actinide species was promoted. While thorium(IV) phosphate alone gave a solid of poor crystallinity (as determined by X-ray diffraction analysis) its deposition onto a pre-formed crystal of $LaPO_4$ resulted in the formation of a new, hybrid crystal - a process termed Microbially- Enhanced Chemisorption of Heavy Metals (MECHM). Using MECHM the removal of both Pu(IV) and Np(V) by *Citrobacter* sp. was promoted under conditions where $LaPO_4$ -free biomass retained little metal [4].

An alternative approach to metal remediation can employ the reductive capability of some bacteria, which can use metals as electron acceptors, using electrons derived from hydrogen (via the activity of hydrogenase and possibly also cytochrome c_3) or via organic electron donors and electron transport chains. The organism *Shewanella putrefaciens* reduced Np(V) to lower valence species but was unable to effect the removal of reduced Np from solution. Conversely, native biomass of *Citrobacter* sp. removed little Np(V) even though phosphate was produced. By concerted use of the two organisms a reduction and simultaneous precipitation technique was developed to remove Np from a very dilute solution against a background of NO_3^- comparable to that occurring in typical wastes.

In addition to metal species some wastes contain tributyl phosphate (TBP), an organophosphorus compound, which, as a general class, are priority pollutants according to EC Directive 76/464, Annex I, List I. New strains of *Pseudomonas* have been identified which can harness phosphotriesterase activity to TBP biodegradation; the liberated inorganic phosphate has been coupled to the removal of uranium from a mixed waste solution using a flow-through immobilized cell reactor [3]. Expression of TBP hydrolase activity appears to be plasmid-associated, in a similar way to the well-established parathion hydrolase enzyme of other species which have been harnessed to the treatment of organophosphorus compound insecticides.

Nuclear decontamination activites comonly use chelating agents to remove traces of residual metal from contaminated plant and equipment. Chelated metal wastes are problematic because although a chelating agent may be biodegradable *per se* it may become recalcitrant in the presence of associated metal ions. Citrate and EDTA are good examples, and the use of new microorganisms in the degradation of these compounds will be described. In the case of EDTA the organisms are new and quite unusual, suggesting the future applications of hitherto ill-documented oligotrophic bacteria in the degradation of metal chelates previously considered as recalcitrant.

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THE RELATIONSHIP BETWEEN THE SEDIMENT MICROBIAL COMMUNITY AND CYCLING OF TRANSURANIUM ELEMENTS

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Introduction. Low level aqueous radioactive effluents have been discharged directly into the Irish Sea from the BNFL Sellafield fuel reprocessing plant since 1952. The early transuranium elements, Np, Pu and Am, contained in the waste have a high affinity for particulate matter and transport of contaminated particles has distributed these elements throughout local estuaries. Areas of intertidal salt marsh within the estuaries are contaminated with $^{239,240}Pu$ and ^{241}Am to a level of 1-2 MBq m^{-2} of each element, and with up to 1 kBq m^{-2} of ^{237}Np. These areas therefore provide ideal sites for the investigation of the biogeochemistry of the early transuranium elements.

Methods. The field site used in this study is a vegetated salt marsh in the Esk Estuary, approximately 10 km south of Sellafield. A previous study [1] at this site identified an annual cycle in the dissolved plutonium concentrations in porewaters and it was suggested that microbially driven redox cycling was responsible. The aim of this present study was to investigate this hypothesis further, examining changes in the microbial community over time and measuring changes in the solubility of Np and Am as well as Pu. Interstitial water samples were collected at monthly intervals throughout a 10 month period from January 1996 from *in-situ* porous cup samplers emplaced at 30 cm depth in the sediment. The Eh, pH and temperature of the solution samples were measured in the field. On each occasion, 3 replicate sediment core samples were also collected.

The interstitial water samples were analysed for Fe and Mn (as redox indicators) and

Na (as a measure of salinity) by conventional instrumental methods (AAS, ICP-OES), for $^{239,240}Pu$ and ^{241}Am by radiochemical separation and α-spectrometry and for ^{237}Np by radiochemical separation [2] and accelerator mass spectrometry (AMS).

The sediment samples were analysed for phospholipid fatty acid (PLFA) biomarkers by chemical extraction, chromatographic separation on silica, then mild alkaline methanolysis, followed by GC-MS determination of the individual methyl esters [3]. Analysis of 6 replicates of a single, homogeneous sediment sample showed that 10 PLFAs could be determined with acceptable precision. The concentration data for these 10 PLFAs were analysed statistically using a multivariate cluster analysis procedure [4].

Results. The statistical analysis of the PLFA data shows that the year can be divided into 5 parts, separated primarily on the basis of total biomass. The structure of the microbial community remains reasonably constant throughout the year and is generally dominated by aerobic organisms. As might be expected, biomass is lowest in mid-winter and increases by a factor of approximately 12 to a late summer maximum, before decreasing again with the onset of winter. Most of the increase in biomass occurs after April.

The transuranium element concentrations follow complex patterns. The seasonality in the $^{239,240}Pu$ concentrations which had previously been observed in 1994 [1] is reproduced in 1996. The $^{239,240}Pu$ activity remains between 2.5 and 3.5 mBq l^{-1} between January and May, then falls steadily to less than 1 mBq l^{-1} in October before increasing again. The ^{241}Am activity concentrations fluctuate in the early part of the year, from 2 mBq l^{-1} in January to 0.5 mBq l^{-1} in March and back to 2 mBq l^{-1} in May, before falling steadily and finally rising at the end of the year, mirroring the behaviour of $^{239,240}Pu$. ^{237}Np activity concentrations also fluctuate in the early part of the year, between 0.4 mBq l^{-1} (January) and 1.1 mBq l^{-1} (March), then decrease steadily after March to 0.1 to 0.2 mBq l^{-1} by May and remain at this level for the remainder of the year.

Discussion. Since the site is always oxic (Eh 180-260 mV), it seems unlikely that redox changes are responsible for the changing concentrations of the transuranium

elements, as previously proposed [1]. This is consistent with the behaviour of Am, which will be insensitive to redox change since it only has access to the (III) oxidation state. Plutonium is expected to be predominantly in oxidation state (IV) [4] while Np will be present as Np(V) species.

The fall in activity concentrations of both Pu and Am in the latter part of the year (April onwards) coincides with the rapid increase in microbial biomass, which could be explained by removal of the highly charged Pu(IV) and Am(III) species through passive accumulation on to the microbial biomass. The increasing activity concentrations of ^{239,240}Pu and ^{241}Am observed at the end of the year could then arise from their release by degrading cells. Np, with its lower charge density, will be much less susceptible to passive uptake, although the generally lower ^{237}Np activity concentrations during the spring and summer months may reflect some accumulation by biomass.

The behaviour of the transuranium elements in the early part of the year (January to March) does not appear to be related to changes in the microbial community. The biomass is relatively low in this period, so binding to microbial material is unlikely to be a dominant control. Moreover, there are times when the concentrations of some elements rise but at the same time the concentrations of others fall, so any changes in the system affect different elements in different ways. It is possible that these changes are microbially driven, but the PLFA technique is not capable of resolving such relatively subtle changes within the community.

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INVESTIGATION OF SUBTERRANEAN BACTERIA IN THE NUCLEAR WASTE REPOSITORY

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Development of science and technology highlighted the importance of microbially induced corrosion (MIC) in radioactive waste repository. These processes should be taken into consideration for the long term safe disposal. Based on preliminary assessment the use of the Permian Boda Claystone Formation in Mecsek Hill area is being considered for high level waste disposal in Hungary. Microbiology programme is to understand how subterranean bacteria will interact with the performance of a future radioactive waste repository. It concerns several major items that may have an influence on the mobility of radionuclides in direct and indirect ways thereby being important for the safety analysis. They are uptake and transport of radionuclide by microorganism, diversity and distribution of subterranean bacteria in special repository environments, environmental limitations, microbial gas production and consumption and microbially induced corrosion of waste canister, including acid production biosorption or accumulation and radiosensitivity. The above factors will be discussed.

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METAL-BACTERIAL INTERACTIONS IN SULPHATE-REDUCING BACTERIAL BIOFILMS

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Bioprecipitation using sulphide produced by sulphate-reducing bacteria (SRB) is an efficient means of removing toxic metals and acidity from sulphate-containing leachates [1,2]. A biofilm reactor system would allow greater process intensification than suspended systems and a reduction in reactor volume. This research was therefore undertaken in order to obtain the necessary basic information on kinetics of growth, substrate utilisation and metal precipitation by SRB biofilms for the development of such bioreactors.

The cultures consisted of a mixed SRB culture enriched in our laboratory [1] and a pure culture, provisionally identified as a *Desulfotomaculum* species, isolated from the mixed culture. Both pure and mixed cultures were subsequently selected for biofilm growth and maintained as biofilms [3]. When mixed SRB biofilms were cultured on polystyrene coupons suspended in a chemostat, the biofilms formed a continuous but uneven layer on the support. They attained a steady state, with no further net growth, after 7 d. They were further cultured in medium containing no added Cd (control), 20 or 200 μM Cd. In the presence of Cd, the biofilm accumulated CdS in large amounts so that the biofilm acquired a yellow colour and, after 14 d incubation with 200 μM Cd, the only elements detectable in energy-dispersive X-ray analysis (EDXA) of the biofilm surface were Cd, S and P. Cd and S were also major components of the 20 μM culture. Uptake of Cd by these biofilms was accompanied by accumulation of both carbohydrate and protein in significantly larger amounts than found in the control (Cd-free) biofilms (Table 1). The accumulation of both polysaccharide and protein correlated significantly with metal uptake (R^2 = 0.60 and 0.65, $p < 0.01$ at 53 d.f.). The deposited Cd was shown by back scattered electron imaging to be located in the surface of the biofilm. This suggested that the main mechanism whereby the SRB biofilms took up Cd from the medium was by formation of insoluble CdS which was then entrapped in the extracellular matrix of the biofilm.

The pure culture biofilms cultured in the same system formed an uneven cover with areas of no growth and areas with chains of cells growing away from the surface. EDXA showed the presence of Cd and S in the pure culture biofilms exposed to both 20 and 200 μM Cd. However, the pure culture took up considerably less Cd from the medium than the mixed culture and also accumulated less carbohydrate and protein than the mixed culture in both control and Cd-containing media (Table 1). Since the sulphide concentrations produced by both cultures were similar (1.0-4.5 mM) and in excess over the total metals in the media (less than 360 μM), the lower Cd uptake by the pure culture biofilm compared to the mixed culture biofilm appeared to be the result of its lower extracellular polymer content and thus lower capacity to entrap solid CdS particles.

These results emphasise both the multi-component nature of the interactions between Cd and SRB biofilms and the significant differences between the interactions of pure and mixed cultures. The major practical advantages of mixed cultures in environmental biotechnology are also highlighted in that, in addition to

process robustness, the mixed culture took up more Cd from solution than the pure culture.

Table 1. Cadmium, polysaccharide and protein accumulation by a mixed and pure sulphate-reducing biofilm culture exposed to varying cadmium concentrations.

Culture	Variable	Control	20μM Cd	200μM Cd	LSD ($p< 0.01$)
Mixed culture	Cd (nmol cm^{-2})	5.12	244.22	780.25	92.08
	Protein (mg cm^{-2})	0.99	1.03	1.18	0.08
	EPS* (μmol cm^{-2})	0.64	0.85	1.76	0.35
Pure culture	Cd (nmol cm^{-2})	14.85	62.83	222.96	26.25
	Protein (mg cm^{-2})	0.13	0.10	0.16	0.06
	EPS* (μmol cm^{-2})	0.23	0.11	0.17	0.06

* EPS carbohydrate in glucose equivalents.

Each value is the mean of 8 determinations with the least significant difference (LSD) being calculated by variance analysis and comprising the residual standard error of the whole data-set for the three treatments multiplied by an appropriate "t" value for the required significance level at 21 degrees of freedom [4].

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BIOACCUMULATION OF LONG-LIVED RADIONUCLIDES BY FRESH-WATER SILT

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The problem of the natural waters contamination by radionuclides appears to be a series problem for most of the countries with the developed nuclear industry. Some traces of fission products are already discovered in the Irish Sea (UK) [1,2], in the ground waters not far from Hanford tanks (USA) [3], in the Rhone-river (France) [4]. The negative radioecological situation also arises in Russia in the "Mayak" plants area (Techa-river, Karachai Lake) [5], and on the Enisei-river around the mining and chemical enterprise [5,6]. For this reason accumulation of radionuclides by bottom sediments of different natural reservoirs is being thoroughly investigated nowadays.
The kinetics analysis of the long-lived ^{99}Tc (VII), ^{237}Np (V), ^{239}Pu (VI) absorption by the samples of the fresh-water silt collected in the lake Beloye Kosino (Moscow region) has shown that the half-time values of the initial content removal from the water-phase form 400 h for Tc, 10 h for Np and less than 1 h for Pu. Reduction, sorption, and hydrolysis are believed to be the main mechanisms for Np and Pu accumulation in the biosystem. Hydrosulfide produced by sulfate-reducing bacteria, that are the component of the silt, is thought to be responsible for the Tc precipitation. Significant difference in Tc bioaccumulation by the silts taken in winter and summer periods, especially in the case of sterilized samples, also confirms the suggestion, that the main mechanism for Tc uptake by fresh-water silt is its reaction by hydrosulfide.

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SESSION IV

ABSTRACTS

Bioremediation of heavy metals and radionuclides by bacteria

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Bacteria, and other microorganisms, have a significant influence on the environmental fate of toxic metals and radionuclides in both aquatic and terrestrial ecosystems with several physico-chemical and biological mechanisms effecting changes in mobility and speciation, and all subject to effects of environmental factors [1,2]. Physico-chemical mechanisms of removal include association with extracellular materials, metabolites and cell walls by, e.g. adsorption, ion exchange, and entrapment which are features of living and dead organisms. In living cells, some physico-chemical processes can be reversible and directly or indirectly influenced by metabolism, and by changing environmental conditions, e.g. pH, E_h, organic and inorganic nutrients, clay minerals, humic materials and salinity. Metabolism-dependent mechanisms of metal immobilization include precipitation as sulphides, transport and intracellular compartmentation and/or sequestration by proteins and peptides. In addition, certain chemical transformations, particularly by reduction, to less soluble forms can result in immobilization. Microbial processes involved in metal solubilization from immobile sources include autotrophic and heterotrophic leaching, complexation by siderophores and other metabolites, and chemical transformations [1,2]. Several microorganism-based biotechnologies, e.g. those involving on biosorption or precipitation, are now receiving interest as potential treatment methods for metal/radionuclide contamination of natural environments and process streams.

This contribution will detail some of the main mechanisms by which bacteria effect changes in metal mobility, and how these may be used in bioremediation, with particular reference to microbially-catalyzed reactions which occur in the natural sulphur cycle which have been integrated in a microbiological process to remove toxic metals from contaminated soils [3]. Here, bioleaching using sulphuric acid produced by sulphur-oxidizing bacteria was followed by precipitation of the leachate metals as insoluble sulphides by sulphate-reducing bacteria. Metal contaminants including Cd, Co, Cr, Cu, Mn, Ni and Zn were efficiently leached from an artificially-contaminated soil: Mn, Ni and Zn were the only target elements which were significantly leached from soil minerals. Pb leaching was slow and remained incomplete over a period of 180 days. Mineral components such as Fe, Ca and Mg were also leached but the eventual reduction in soil mass was only approximately 10%. An industrially-contaminated soil was also efficiently leached and approximately 69% of the main toxic metals present, Cu, Ni and Mn, were removed after 175 days. The leachate which resulted from the action of sulphur-oxidizing bacteria on contaminated soil was stripped of metals using an anaerobic bioreactor containing a mixed culture of sulphate-reducing bacteria which precipitated soluble metal species as solid metal sulphides. More than 98% of the metals were removed from solution with the exception of Mn, Ni and Pb where 80-90% were removed. The metal content of the resultant effluent liquor was low enough to meet European criteria for discharge into the environment.

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NOVEL TECHNOLOGY FOR CLEANUP OF CHLORINE ALKALI ELECTROLYSIS PROCESS WASTE WATER USING MERCURY RESISTANT BACTERIA

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Mercury compounds are among the most toxic substances on earth. Local pollution hot spots can easily be transported to adjacent ecosystems, enter the food chain and accumulate by several orders of magnitude in top predators including man. Therefore, the prevention of mercury pollution and the remediation of old waste is a task of high priority within the European community. To this end, technologies are needed which specifically remove low levels of highly toxic mercury compounds from large volumes of waste water at a reasonable price. The discharge limit for industrial waste water is 50 µg/l. For drinking water, less than 1 µg/l is permitted. The most important source for mercury contaminated waste water is the chlorine-alkali electrolysis process. In particular old plants (built before 1970) produce waste water which consists almost exclusively of several species of mercury and salts.

Microorganisms during their evolution have developed resistance mechanisms against mercury and its compounds. They can, however, not destroy mercury, but only transform it from a highly toxic compound to a compound with much lower toxicity. The most widespread microbial resistance mechanism known so far is reduction of ionic mercury to metallic mercury, which is a cofactor dependent transformation catalysed by the intracellular mercury reductase enzyme. For regeneration of the cofactor (biologically active hydrogen in the form of NADPH) the microorganisms need carbon as a source of energy. The reductase enzyme is coded by the *merA*

gene of the mercury resistance operon, the so called *mer* operon, which is found in both Gram negative and Gram positive bacteria and shows a high degree of structural and sequence homology. It is often located on transposable elements and can occur either on the chromosome or on plasmids.

We have isolated mercury resistant bacteria from chlorine alkali waste water and contaminated sediments, identified them by physiological tests and 16S rDNA sequencing and shown the presence of the *merA* gene [2, 3]. These organisms transform mercury even at low concentrations completely, quickly (within minutes) and without accumulation of side products. They always produce metallic mercury, which precipitates above its solubility concentration (≈ 50 µg/l in water). If the bacteria are immobilized on solid supports, the precipitated metallic mercury is held back within the microbial biofilm, from which it can be separated and recovered at the end of the process after several months [1, 4]. By scanning electron microscopy and EDX analysis of bioreactor carrier material it could be shown that mercury is deposited as little spheres of up to 8 µm diameter, preferentially close to the reactor inflow. Therefore, active microbial mercury reduction using *merA*$^+$ bacteria has the potential for cleanup of contaminated waste water.

We proceeded from pure mercury chloride solutions to chlorine alkali waste water in a stepwise fashion, using model waste water with increasing concentrations of potassium chloride as an intermediate. Thus we could show that mercury reduction activity in suspension and in microbial biofilms is not inhibited up to 20 g NaCl/l. We also demonstrated effective microbial mercury removal from chlorine alkali waste water from several companies. Total mercury concentrations in the waste waters tested ranged from 1.5 to 8.0 mg/l, effluent concentrations at optimum reactor performance were appr. 200 µg/l, representing a cleanup efficiency of 87 - 97 %.

A pilot plant for mercury removal from chlorine alkali electrolysis waste water in half technical scale was developed based on biochemical engineering studies on fixed bed reactors in volumes up to 1 l. The pilot plant has a bed volume of 1 m^3 and will be able to treat up to 4 m^3 water/h. Incoming waste is neutralized and supplemented with saccharose before it enters the bioreactor. The reactor is operated at ambient temperature under non-sterile conditions. Inexpensive porous ceramic materials, e.g. Biopor and/or spotted lava will be used as carriers. Mercury concentration, pH,

chlorine gas and temperature are continuously monitored in the inflow and controlled to stay within the range acceptable for the microbial biofilm. Measurement of redox potential and pO_2 will provide information on the activity of the bacteria. Axial sampling ports will allow off-line measurement of mercury concentration and speciation and allow sampling of biofilm material. To fulfil industrial discharge limits, the pilot plant is equipped with an activated carbon filter after the bioreactor outflow. A laboratory mounted on a four-wheel drive van (Mobile Laboratory of the GBF) and equipped with the necessary instrumentation will be used to monitor the reactor performance *on site*.

To estimate the efficiency and operation period of the 1 m^3 demonstration reactor, a capacitiy of 40 g mercury per l l of bed volume can be assumed. On the basis of a waste stream concentration of 2 mg/l of mercury ions a total waste volume of 20.000 m^3 can be treated by the bed. If a waste stream volume of 3 m^3/h is assumed, representing a nominal residence time of 20 min, this means the process can be operated for more than 200 days. The concentration factor is 20.000, i.e. mercury contained in 20.000 m^3 will be concentrated to 1 m^3. This calculation based on data verified in laboratory scale suggests an enormous capacity of the microbial mercury transformation process.

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INTERACTION OF OXYANIONS WITH MICROBIAL BIOMASS.
THE CASE OF Cr AND Se

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The interaction of Cr(VI) and Se(IV) with selected microbial biomass has been examined. Biosorption of Cr (VI) on inactive microbial biomass is pH dependent and only at pH values below 2 sorption of CrO_4^{2-} can be considered noticeable.

It has been also observed that part of Cr(VI) present in the solution is reduced to Cr (III) by a redox reaction possibly by oxidation of cell components. Biosorption of the reduced Cr(III) also contributes to the removal of total Cr from the solution. Experiments with metabolically active microbial biomass has revealed that reduction of Cr (VI) to Cr (III) is facilitated, possibly due to contribution of Cr (VI) as an electron acceptor.

Metabolically mediated reduction of Se(IV) to elemental Selenium has also been observed for bacterial strains tested. The colourless solutions of Se(IV) become red after inoculation of the nutrient medium with bacterial culture indicating the formation of red amorphous elemental Se.

REDUCTION OF HEXAVALENT CHROMIUM BY MIXED CULTURE SULPHATE-REDUCING BACTERIAL BIOFILMS

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At low concentrations, chromium is an essential trace metal for human and animal nutrition. However, Cr and its associated compounds are widely used in industry and the extent of wastewater discharge has led to some serious pollution problems [1,2]. Cr has a range of oxidation states each with different solubilities, bioavailability and therefore microbial toxicity. In the highest oxidation state, Cr exists as two highly soluble and toxic oxyanionic forms: chromate (CrO_4^{2-}) and dichromate ($Cr_2O_7^{2-}$) both of which are mutagenic, carcinogenic and teratogenic [1]. Furthermore, Cr is an analogue of the sulphate anion (SO_4^{2-}) having the same charge and a similar physical size, and therefore has serious environmental and health implications should it compete with sulphate uptake and metabolism. However, in the most stable, trivalent oxidation state, chromium exists mainly as an insoluble amorphous hydroxide, $Cr(OH)_3$ [2]. Reduction of the highly soluble chromate oxyanion to the more stable, less toxic insoluble form of Cr(III) therefore represents a means for remediation of this toxic pollutant and perhaps a mechanism which enables microbial survival [3].

This work has investigated the reduction of hexavalent chromium using sulphate-reducing bacterial biofilms [4,5]. Sulphate-reducing bacteria are obligate anaerobic heterotrophs which couple the oxidation of their carbon/energy source to the dissimilatory reduction of sulphate [6]. The sulphide produced can be used to reduce and/or precipitate metals in insoluble forms [7,8]. Biofilms are now recognised as being an important mode of growth for sulphate-reducing, as well as other, bacteria, conferring resistance to environmental stress factors such as dehydration and toxic substances including metals and biocides [9]. Biofilms also have the ability to influence the chemistry of metal and organic pollutants through various interactions which include sorption, reduction and precipitation [10,11].

Mixed culture SRB biofilms utilising lactate as the carbon/energy source were used in experimental work with the reduction of 500 μM Cr(VI) being investigated over a 48 h period. It was found that rapid reduction of chromate occurred within the first 10 h of incubation, with a total of 88% being removed from solution by the end of the experiment. Mass balance studies indicated that the majority of the chromium was precipitated, and there was only negligible sorption of Cr onto the biofilm. Monitoring of the metabolism of the sulphate-reducing bacterial biofilms over this 48 h period showed that this concentration of chromate did have inhibitory effects. In the absence of chromate, sulphate reduction and sulphide production proceeded unhindered, with sulphate reduction and lactate utilisation occurring within the first 10 h. In the presence of chromate however, very little sulphate was reduced and low levels of sulphide were therefore produced. Rates of lactate utilisation were also slower than the controls with less acetate being produced. The significance of these findings and their potential application to bioremediation will be discussed.

Acknowledgement:
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INVITED

AND

CONTRIBUTED ORAL

PRESENTATIONS

SESSION V

ABSTRACTS

GENOMIC ORGANIZATION OF ACIDOPHILIC CHEMOLITHOTROPHIC BACTERIA.

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The genomic organization of acidophilic chemolithotrophic bacteria belonging to the genus *Thiobacillus*, *Thiomonas* and *Leptospirillum* was studied using pulsed field gel electrophoresis techniques (PFGE). The electrophoretic analysis of intact DNA prepared from different strains showed that all have a circular chromosome, with sizes ranging from 1.5 Mb for *Leptospirillum ferrooxidans* sp. isolated from the Tinto River, to 3.8 Mb for *Thiomonas cuprina* DSM5495, the largest in this study. The number of extrachromosomal elements present in these strains varied from none, as observed in several isolates of *Leptospirillum ferrooxidans*, to five in *Thiobacillus thiooxidans* ATCC 8085. The mixotroph *Thiomonas cuprina* DSM 5495 was found to have a linear 50 Kb megaplasmid which was inducible when the bacteria was grown in chemolitrophic conditions. Low-frequency restriction fragment analysis of different acidophilic chemolithotrophs and related species was carried out by PFGE to determine macrorestriction patterns from rare cutters, which can be used for taxonomic identification, genome size determination and physical map generation. A top-down approach produced high resolution maps for the circular chromosomes of *Thiomonas cuprina* DSM 5495 and *Thiobacillus ferrooxidans* ATCC 21834. The use of homologous and heterologous probes were used for the generation of low-resolution genetic maps for these fastidious microorganisms.

Molecular physiology of heavy metal resistance in *Alcaligenes eutrophus* CH34

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Alcaligenes eutrophus strain CH34 contains at least seven determinants encoding resistances to toxic heavy metals, located either on the bacterial chromosome or on one of the two indigenous plasmids pMOL28 (180 kilobase pairs (kb)) and pMOL30 (238 kb). One of these, the *czc*-determinant of plasmid pMOL30, mediates inducible resistance to Co^{2+}, Zn^{2+} and Cd^{2+} in *A. eutrophus* . The products of the genes *czcA*, *czcB*, and *czcC* form a membrane-bound protein complex catalyzing an energy-dependent efflux of these three metal cations. The mechanism of action of CzcCBA complex [2] is that of a proton/cation antiporter. CzcA has been purified to homogeneity and reconstituted into proteoliposomes; it is the central proton/cation antiporter of the Czc system and is driven by the pH gradient. Conserved amino acids were mutated in *czcA* and resistance and transport mediated by the resulting CzcA derivatives was analysed. Together with a topological analysis of CzcA by reporter fusion scanning, this allows a detailed model of CzcA working as a two-channel pump.

The *czc* determinant is regulated by at least 7 proteins: CzcN, CzcI, CzcD, CzcR, CzcS, CzcE and a proposed extracellular function sigma factor. Regulation of the *czc* determinant was studied on the level of transcription with Northern hybridization, dot blots [1], primer extension, RT-PCR and semiquantitative RT-PCR in wildtype cells, using a *czcC-lacZ-czcBA* reporter strain, and several mutants of both strains. Moreover, CzcR was purified and its binding site was identified [4]. The data obtained can be explained with a complex regulatory pattern of cobalt-zinc-cadmium homoeostasis in this bacterium.

On plasmid pMOL28, two inducible metal resistance determinants are located adjacent to each other: the *cnr* determinant encodes resistance to Co^{2+} and Ni^{2+} , and physiologically is based on metal cation efflux. The *chr* determinant gives resistance to chromate. The mechanism of chromate resistance is reduced accumulation of chromium (Nies and Silver, 1989) , but chromate efflux has not been demonstrated. An interaction between induction of the *chr* resistance determinant, of the genes upstream or downstream of *chr*, chromate reduction, chromate accumulation and the sulfate concentration of the growth medium was demonstrated [3].

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HEAVY-METAL INDUCED GENE EXPRESSION IN SOIL BACTERIA

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The occurrence of elevated concentrations of heavy metals in the environment represents a challenge for soil bacteria. On the one hand, certain metal ions, such as copper and zinc, play important roles in diverse metabolic processes. On the other hand, these metals are toxic to cells when present in high concentrations. Moreover, other metals, including cadmium, mercury and lead, have no known biological function and are poisonous to cells even in low concentrations.

We used a genetic approach to identify mechanisms soil bacteria employ to cope with elevated concentrations of heavy metals. A random mutagenesis was carried out using a common soil bacterium, *Pseudomonas fluorescens* ATCC strain 13525. For the mutagenesis, a transposon Tn*5* derivative was used, which carries a promoterless *lacZ* gene as a reporter at one of its end (Tn*5*-B20, [1]). The *lacZ* gene encodes the enzyme beta-galactosidase. If the transposon inserts downstream of a promoter in the correct orientation, then a transcriptional fusion is generated and the level of expression of the targeted promoter can be easily determined by measuring the beta-galactosidase activity. Convenient methods to determine the enzyme activity of beta-galactosidase are available.

Five thousand *P. fluorescens* mutant strains, all carrying the reporter gene transposon at different locations in the genome were generated and exposed to elevated concentrations of cadmium chloride. The mutant strains were screened for differential gene expression in the presence of cadmium. Thirteen strains were identified that displayed increased gene expression, whereas four strains showed decreased gene expression in the presence of cadmium. We analyzed whether this response was specific for cadmium, or a response to metal ions in general. Some of the genes

identified in our study responded in a similar fashion to elevated concentrations of copper and zinc. Thus, these genes may play a role in the general metal homeostasis of the cell. Moreover, one of the genes did not only show increased gene expression in the presence of metals, but also in the presence of solvents (ethanol). We conclude that this gene may play a role in the general stress response of the cell.

Our hypothesis is that genes which show increased gene expression in the presence of heavy metals are involved in helping the organism to cope with these metals. Thus, strains with mutations in these genes should show a reduced fitness when grown in the presence of metals. In fact, one of the transposon-carrying mutant strains showed a reduced growth rate in the presence of cadmium and zinc when compared to the growth rate of the wild-type strain. Cloning of the targeted gene and subsequent determination of the DNA sequence revealed that in this instance the transposon had inserted in an operon that shared high similarity with the *czc* operon of *Ralstonia (Alcaligenes) eutropha*. In this organism, the *czc* operon confers a high level of resistance to cobalt, zinc, and cadmium by encoding an efficient cation-proton antiporter that frees the cell from metal ions [2]. It is interesting to note that in contrast to *R. eutropha*, the strain used in our studies had not been isolated from a metal polluted site, but nevertheless seems to contain similar genes to defend itself against toxic metal ions. In summary, our approach can aid in the identification of some of the strategies soil bacteria have developed to cope with elevated concentrations of heavy metals.

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CATALYSIS PERFORMED BY A BACTERIAL GLUTAMINE SYNTHETASE IN THE PRESENCE OF IONS OF *s*-, *p*- AND *d*-ELEMENTS

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Glutamine synthetase (GS) is one of major enzymes of nitrogen metabolism in bacteria [1]. This enzyme contains two metal ions in the active site, and like most synthetases is activated *in vivo* usually by Mg^{2+} or Mn^{2+}. Since there are contitions allowing formation of bihomonuclear or biheteronuclear complexes of metals with GS, we studied biosynthetic (1) and transferase (2) reactions of this enzyme isolated from the soil nitrogen-fixing bacterium *Azospirillum brasilense* Sp. 245 at two pH levels, one (pH 6,2) is optimal for functioning of GS in the presence of Mn^{2+}, another (pH 8,0) is optimal when Mn^{2+} is replaced by Mg^{2+}[2].

L(D)-Glu + NH_4^+ + ATP => *L(D)*-Gln + ADP + P_i + H_2O (1)

L-Gln + NH_2OH + ADP =$\underline{AsO_4^{3-}, AsO_3^{3-}}$=> γ-GGK + AMP + P_i + NH_4^+ + H_2O (2)

The physiological ions in the assay mixtures were replaced by almost any s-, p- and d-metal ions of the periodic table (excluding alcaline metals), as well as by their mixtures with manganese and magnesium. The results are shown on Fig. 1 and 2. We found that heteronuclear complexes are really formed during interaction of GS with metal ions and these species could be more or less active than homonuclear analogs. The maximal reaction velocity was observed for complexes MgCoGS. d^{10}-elements (Zn, Cd, Hg) in heteronuclear complexes decrease catalytic activity of the enzyme as compared with its activity in the form of homonuclear complex.

It was also shown, that if the metal radius is greater than 1,26–1,35Å, then interaction of metal ions with the active site of GS is greatly influenced by steric effects.

Finally, we assume that topology of the coordination sphere is not changed durung the course of the reaction and transferase reaction seems to use only one metal ion in the active site.

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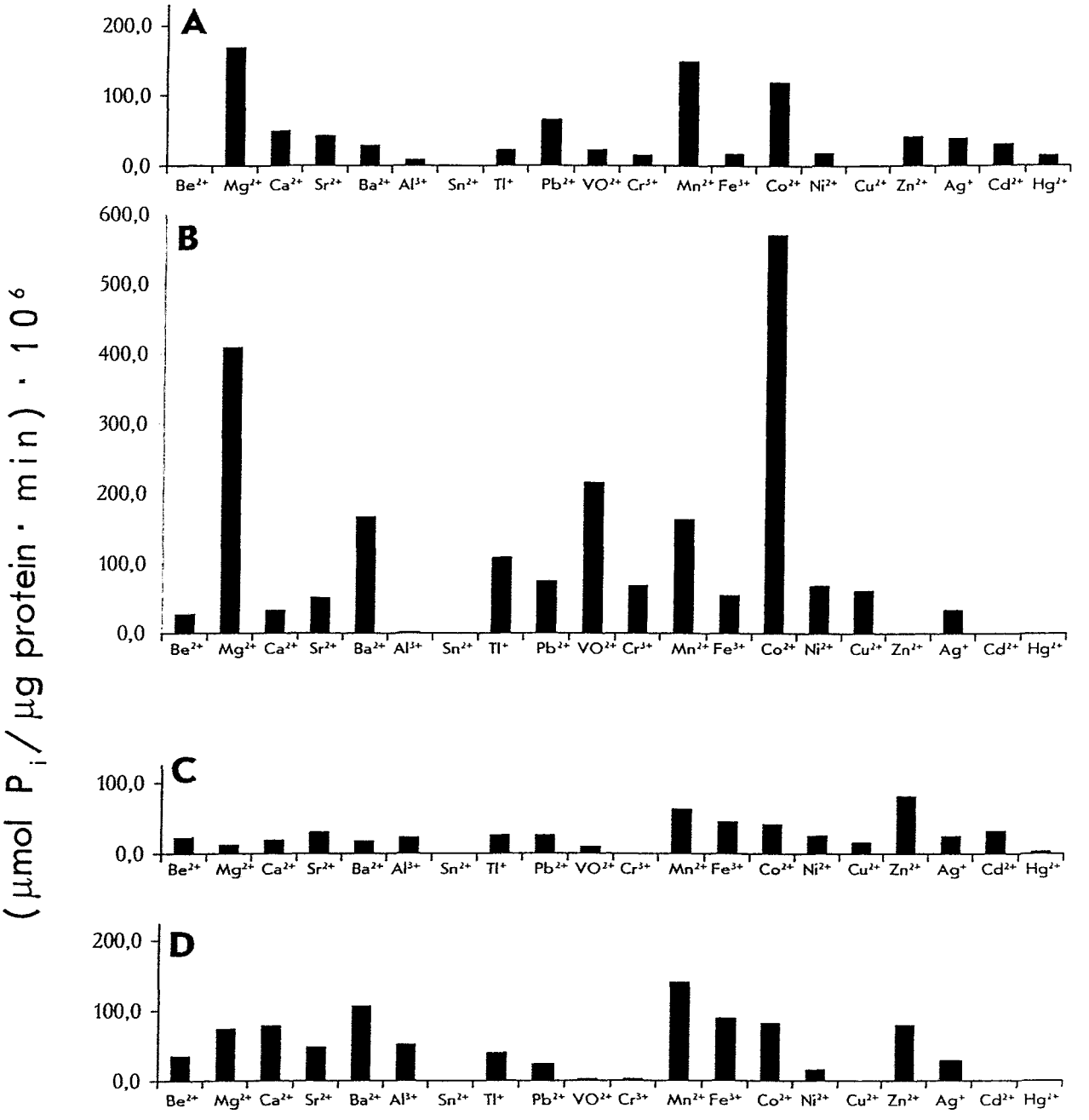

Fig. 1. A — GS activity in the biosynthetic assay at pH 8,0 in the presence of 2 мМ Me^{m+}; **B** — the same, but in the presence of 2 мМ Mg^{2+}+2 мМ Me^{m+}; **C** — GS activity in the biosynthetic assay at pH 6,2 in the presence of 2 мМ Me^{m+}; **D** — the same, but in the presence of 2 мМ Mn^{2+}+2 мМ Me^{m+}

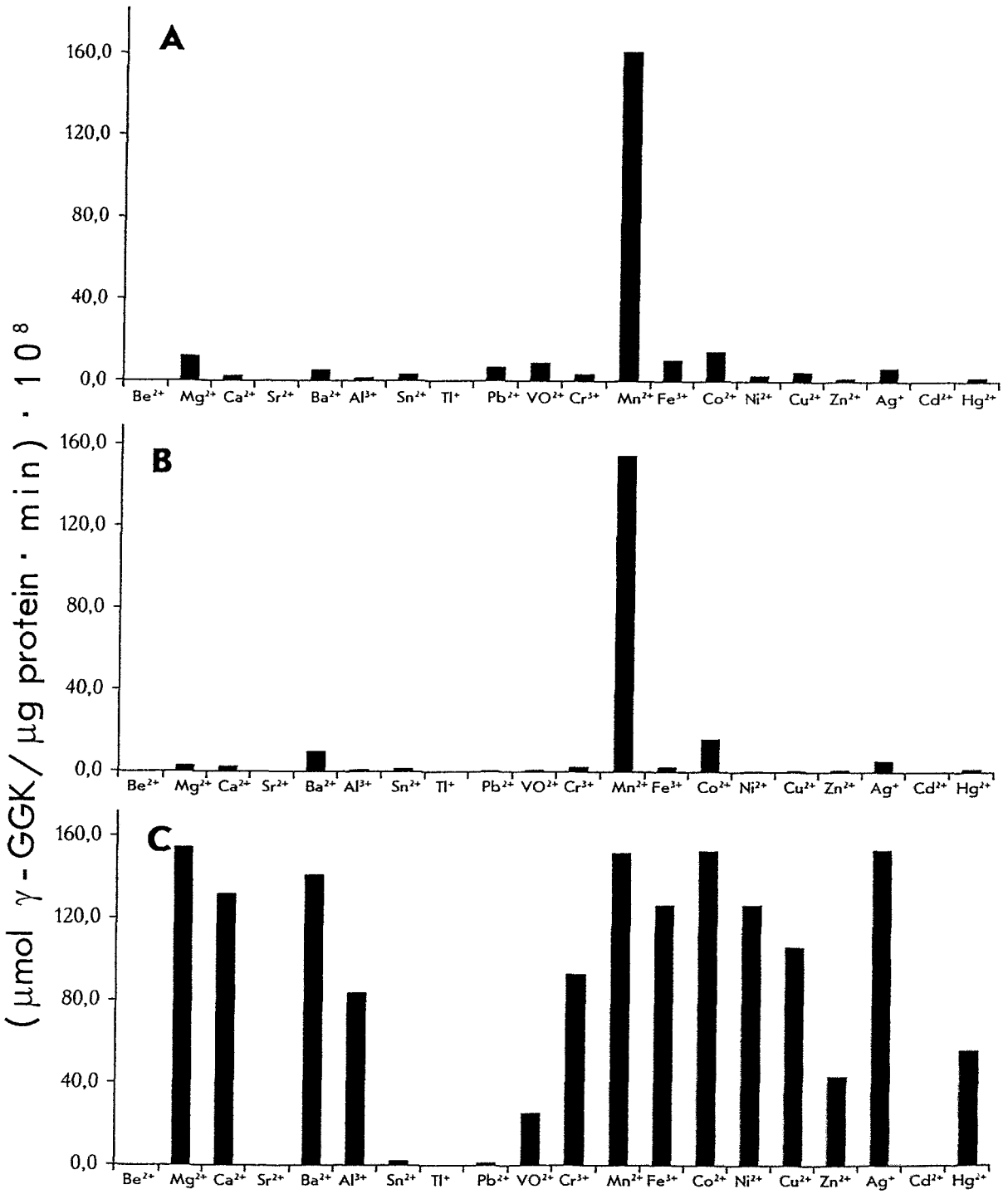

Fig. 2. GS activity in the transferase assay. **A** — in the presence of 2 мМ Me^{m+}; **B** — in the presence of 2 мМ Mg^{2+}+2 мМ Me^{m+}; **C** — in the presence of 2 мМ Mn^{2+}+2 мМ Me^{m+}

APPLICATION OF MICROBIAL TECHNETIUM REDUCTION TO THE TREATMENT OF NUCLEAR WASTE

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Technetium (^{99}Tc; half-life = 2.1 x 10^5 years), a fission product of ^{235}U, is a problematic component of some wastes from the nuclear fuel cycle. This element can exist in multiple oxidation states from +7 to -1 but the most stable state is +7, typified by the pertechnetate ion (TcO_4^-). In this form ^{99}Tc shows poor ligand complexing capabilities. As a consequence,Tc(VII) is difficult to remove from nuclear waste streams and, if released, it is highly mobile in the environment. For these reasons there is considerable interest in the development of novel biotechnological approaches to treat Tc(VII) contaminated waste.

Microbial reduction of TcO_4^- has been proposed as a method to treat Tc(VII)-contaminated effluents due to the low solubility of reduced Tc (for example Tc(IV) and Tc(V)). Lloyd and Macaskie [1] demonstrated enzymatic reduction of Tc(VII) by resting cells of the iron-reducing bacteria *Shewanella putrefaciens* and *Geobacter metallireducens* using a novel phosphorimager-based technique. Subsequent studies have shown that the ability to reduce Tc(VII) is not exclusive to iron-reducing bacteria, but is catalyzed also by resting cells of *Escherichia coli* [2] and the sulfate-reducing bacterium *Desulfovibrio desulfuricans* [3].

Anaerobically (but not aerobically) grown cells of *E. coli* were able to couple the oxidation of formate or hydrogen to Tc(VII) reduction [2], with reduced Tc precipitated within the cell. Using physiological and genetic approaches, the enzyme responsible for Tc(VII) reduction was identified as hydrogenase 3, a component of the formate hydrogenlyase complex [2]. Resting cells of *E. coli*, immobilized in a membrane bioreactor, were used to remove the radionuclide continuously from a challenge solution supplemented with formate or hydrogen as electron donors for metal reduction [4]. Formate- and hydrogen-dependent Tc(VII) reductase activity was also detected in resting cell suspensions of the sulfate-reducing bacterium (SRB) *Desulfovibrio desulfuricans*. Proton-induced X-ray emission studies, in combination with X-ray absorption spectroscopy, confirmed precipitation of a reduced, cell-associated oxide of Tc [3]. The periphery of the cell was identified as the site of deposition by transmission electron microscopy [5]. Removal of Tc(VII) was more efficient when the SRB was immobilized in the membrane bioreactor, compared to a regulatory mutant of *E. coli* with increased activities of hydrogenase 3 or the parental *E. coli* strain. In addition, treatment of Tc(VII) against a high background of nitrate was possible only with the SRB.

Although the genetics and physiology underlying bacterial Tc(VII) reduction are now well understood, the indirect effects of bacterial metabolism on environmental mobility of Tc have received comparatively little attention. Reduced products formed during anaerobic respiration on Fe(III), sulphate or humic acids can act as 'electron shuttles' which reduce Tc(VII), potentially promoting sorption of the radionuclide onto soils. Recent work on the indirect effects of anaerobic processes on Tc speciation and mobility will be presented, in addition to an overview of the genetics, physiology and biotechnological application of enzymatic Tc(VII) reduction.

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POSTER SESSION

ABSTRACTS

ROLE OF THE IONS OF *f*-ELEMENTS IN BIOLOGICAL SYSTEMS. ELECTROCHEMICAL AND COLORIMETRIC STUDY ON INTERACTION OF A BACTERIAL GLUTAMINE SYNTHETASE AND BOVINE SERUM ALBUMINE WITH LANTHANOIDES AND URANYL (VI) IONS

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It is known that many of the lanthanoides and some actinoides have bigger abundance in the Earth's crust than such biologically important elements as iodine and selenium. Besides, these elements are capable of efficient catalyzing of hydrolysis of different organic oligophosphates. In spite of these facts, there were no biochemical processes observed that explicitly require *f*-elements. In this connection we undertook a study of catalytic behaviour of glutamine synthetase of the soil nitrogen-fixing bacterium *Azospirillum brasilense* Sp. 245 in the presence of ions of lanthanoides and uranyl (VI) ion. Also, the intrinsic ATP-hydrolytic activity of these ions and their complexes with bovine serum albumine (BSA) had been investigated (Fig. 1).

Based on the results obtained we could split the lanthanoid series into five regions where mixed complexes of Ln with protein molecules and low-molecular compounds remain isostrucrural and/or isostoichiometric. These regions are La — Pr, Nd — Eu, Gd — Tb, Dy — Ho and Er — Lu. Apart from common considerations of coordination chemistry, one of the changes in the series (Dy) is assumed to be due to decreasing the ionic radius of the element down to 0,88Å and changing the state of ATP from tridentate to bidentate [1]. It seems also that crystallographic behaviour of Ln in their complexes with proteins along the series is similar to that of their ordinary inorganic compounds [2,3] and complexes with macrocycles [4].

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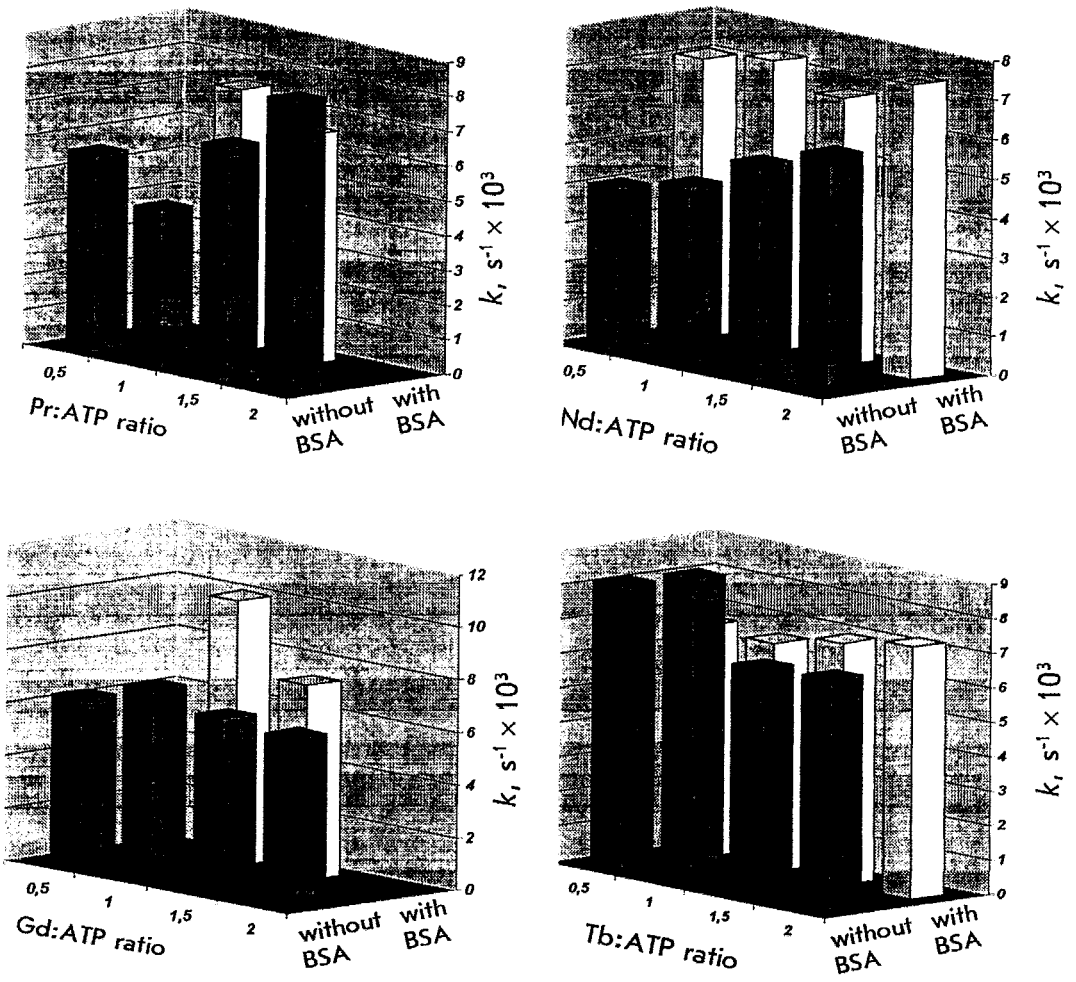

Fig. 1. Relations between hydrolysis constants and Ln:ATP ratio in the presence and absence of BSA.

SOLUBILIZATION OF ZINC PHOSPHATE BY A STRAIN OF *PSEUDOMONAS FLUORESCENS* ISOLATED FROM A FOREST SOIL

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Zinc is a micronutrient required by living organisms, but in many instances, it can exhibit toxic effects at relatively low concentrations, and increased circulation of zinc in the biosphere due to anthropogenic emissions may represent a source of toxic stress. Microorganisms play an important role in the biogeochemical cycling of this element through both bioprecipitation and leaching. Bioprecipitation can result from the action of sulphate-reducing bacteria or as a consequence of specific metal resistance mechanisms [1,2]. Leaching or mobilization can result from the release of chelating agents or from a locally increased proton activity [2,3]. In this work, a strain of *Pseudomonas fluorescens*, able to solubilize zinc phosphate, was isolated from a forest soil. When glucose was provided as the carbon source, colonies of the microorganism produced clear zones on solid medium incorporating zinc phosphate. Both an increase in the H^+ concentration of the medium, probably a consequence of ammonia assimilation, and the production of gluconic acid were responsible for the solubilization of zinc phosphate (Fig.1), and high concentrations of gluconic acid were produced when *P. fluorescens* 3a was cultured in liquid medium in the presence of zinc phosphate.

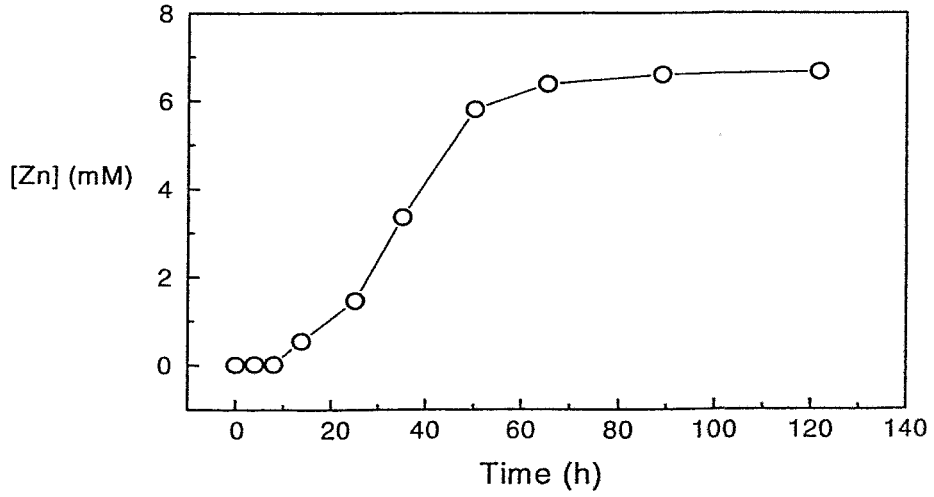

Fig. 1. Soluble Zn^{2+} concentrations in cultures of *P. fluorescens* 3a supplemented with 5 mM zinc phosphate.

Whilst no evidence of zinc chelation was obtained in our experiments, under some conditions gluconic acid is reported to solubilize metals by the formation of chelates. It was observed that the increased Zn^{2+} concentration caused by the solubilization process resulted in the manifestation of toxic effects on the culture. The lack of gluconic acid overproduction in cultures of *P. fluorescens* 3a which were not amended with zinc phosphate suggests that at least some of the glucose oxidation required for

the zinc phosphate solubilization occurred as a result of the toxic stress caused by the high Zn^{2+} concentration [4].

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HEAVY METAL BIOREMEDIATION USING ACIDOPHILIC FUNGI

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Isolated fungi from the Tinto River (pH 2-2.5 and high concentration of heavy metals) were exposed to different metal cations such as Ag, Hg, Cu, Zn, As, Cr, Ni and Cd. The fungal strains were grown in the presence of the different heavy metal solutions with concentrations ranging from 0.1 mM to 400 mM. Many acidophilic fungi exhibited a rather complex pedigree of metal resitance, which is probably related to the extreme conditions in which develop. Several of the strains resistant to specific cations showed the property of sequestering the metal from the growth media, reducing in some cases 90% of the total cation present in the solution. Table 1 shows the metal sequestering capabilities of some of the acidophilic fungi isolated from the Tinto River. The removal specifity observed for some strains could be the base of future applications in bioremediation and alternative metal purification technologies for metallurgy. We are currently studying the sequestering process during the exposure of some of the resistant strains to industrial metal solutions (mixture of metals) to evaluate their real biotechnological potential.

Fungal strain	Metal concentration	% sequestered metal
Aspergillus sp. P38	10 mM Cr	36.7
Penicillium sp. Y25	200 mM Zn	93.8
Trichoderma sp. O8	1 mM Ag	51.8
Scytalidium sp. P65	10 mM Cd	90
Penicillium sp. P22	50 mM As	31.6

Table 1: Metal resistance and percentage of removal using different fungal isolates.

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P 4

THE BIOLOGICAL REMOVAL OF Zn AND Ni FROM A WASTE WATER SUPPLEMENTED WITH INDUSTRIAL CARBON SOURCES.

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For the development of a biological system for the removal of heavy metals a suitable supplementation with nutrients (type and concentration) is crucial from an economical and technical point of view.

In the present work a rinsing water from galvanic industry containing Zn (9.5 mg/L) and Ni (9.0 mg/L) was supplemented with two kinds of carbon sources, i) synthetic C-sources: acetate and lactate, ii) complex mixtures of economic, industrial C-sources: AQUAGUARD (BP Chemicals Ltd., UK), Acetol 20 (Poll GmbH, Germany). The concentrations of the carbon sources were varied from 1 to 1000 mg carbon per litre.

For the inoculation a mixture of four biosorbing and/or bioprecipitating bacteria was used. The effect on growth (optical density) and on metal removal (residual metal concentration) was investigated. Up to a concentration of 1 g carbon per litre no growth limitation was found due to other nutrients already present in the waste water. In contrast to lactate and AQUAGUARD, acetate and Acetol 20 showed an inhibitory effect at a concentration of 1 g carbon per litre.

An increase of the pH value was observed in the presence of each carbon source.

The amount of the metal removal was related to the concentration of the supplemented carbon. With all carbon sources besides Acetol 20 a metal removal of 90% to 95% was achieved in a concentration range of 60-250 mg carbon per litre. An overdosing of acetate and lactate (more then 250 mg carbon per litre) led to a decreased Ni-removal.

This study confirms the importance of an appropriate selection of the type and the concentration of the carbon source for an optimal process of heavy metal removal.

P 5

ACCUMULATION OF METALS BY BACTERIOGENIC IRON OXIDES IN A SUBTERRANEAN ENVIRONMENT

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Bacteriogenic iron oxides (BIOS) and groundwater samples were collected at depths ranging from 66 to 432 m underground at the Äspö Hard Rock Laboratory near Oskarshamn, Sweden. The twisted iron oxide-encrusted stalks of the lithoautotrophic ferrous iron oxidizing bacterium Gallionella ferruginea were prominent in the BIOS samples.

A wide variety of heterotrophic bacteria, including stalked forms resembling Caulobacter or Hyphomicrobium species, were also present.

Energy dispersive x-ray spectroscopy, selected area electron diffraction, and X-ray diffraction analyses confirmed that the BIOS samples only contained poorly ordered (amorphous) hydrous ferric oxide. Inductively coupled plasma emission spectroscopy revealed iron oxide contents that varied from 60 to 90 % on a dry weight basis. Metal concentrations in filtered groundwater ranged from about 10 mM for Na to 10-4 mM and lower for Co, Cu, Cr, and Zn.

Intermediate concentrations were recorded for Fe and Mn (ca. 10-2 mM). Solid phase metal concentrations in the BIOS spanned the 10-2 to 10-5 mmole/kg range. Metal distribution coefficients (Kd values), calculated as the ratio between BIOS and dissolved metal concentrations, revealed solid phase enrichments that, depending on the metal, extended over 4 orders of magnitude. At the same time, however, a distinct decreasing trend in Kd values with increasing iron oxide content was evident for each metal, implying that metal uptake was strongly influenced by the relative proportion of bacterial organic matter in the composite solids. Based on the metal accumulation properties of the BIOS, an important role can be inferred for intermixed iron oxides and bacterial organic matter in the transport and fate of dissolved metals in groundwater systems.

P 6

EFFECT OF TOXIC Cu CONCENTRATION ON THE GROWTH, SYMBIOTIC N2-FIXATION AND ION EXCHANGE ACTIVITIES OF WILD STRAIN AND PLASMID-CURED DERIVATIVES OF *Rhizobium* sp. EFFECTIVELY NODULATING BLACK LOCUST /*Robinia pseudoacacia* L./

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Introduction The plasmid DNA found in the fast- growing rhizobial strains are responsible for determination of some physiological activities of cells like symbiotic N_2 fixation, stress tolerance reaction to different environmental disorders, cell membrane permeability determinants[6,8]. N_2 fixing properties of the *Rhizobium*-legume symbiotic relationships are very sensitive to the changes in the abiotic environment, but little information is available about the involvement of rhizobial plasmids in determination of the stress tolerance reaction of both, free-living and nodulating organisms[1,2].

The aim of this work was to study the plasmid profile of the indigenous isolate *Rhizobium sp.* DC-2, effectively nodulating *Robinia pseudoacacia* L. plants and to elucidate the role of these plasmids in the determination of the growth, ion exchange and symbiotic N_2 fixation of wild and plasmid-cured strains of *Rhizobium sp* DC-2 grown in the present of toxic Cu concentrations in the medium.

Material and methods Plant saplings of *Robinia pseudoacacia* L. clone Svishtov were grown from the root cuttings in the pots containing 15 kg of mixture /luvisol - sand - perlite = 1:1:1/ in the green house conditions [3]. The saplings were inoculated with bacterial suspension of *Rhizobium sp* DC-2 containing 10^8 cells per ml. The pots were supplied with the 200 ppm Cu during the stage of emergence. The plasmid contents of the wild type and cured derivatives of *Rhizobium sp.* isolate DC-2 were determined by a modified Erckhart method [5]. The plasmid curing and isolation of derivatives was performed by the method of Zurkowski and Lorkiewicz (1978). Mineral contents of plasmid-cured and wild strain of Cu stressed 48 h YEM broth culture of *Rhizobium sp.* DC-2 and the nodules of 90 days old plants were analysed by the atomic absorption spectrometry method [3]. The rate of acetylene reduction /ARA/ and ureide content of nodules were determined [3].

Results and Discussion. The wild strain *Rhizobium sp* DC-2 contained very large plasmid (> 1700 kb), as well two additional plasmids in size about 450 and 160 kb, respectively as shown in Fig1 (Fig.1, lanes 2,3 and 4). The derivatives of the

Rhizobium sp. DC-2 have shown a loss of all plasmids after curing (Fig.1, lanes 4,5 and 6), and they had Nod$^-$ phenotype in greenhouse experiments.

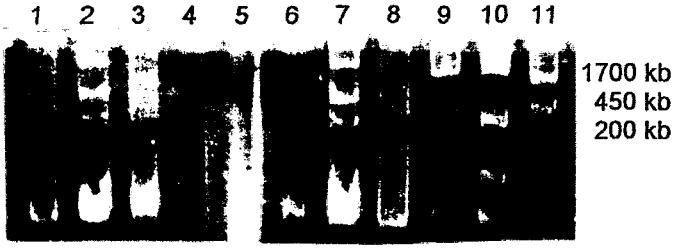

Fig 1. Plasmid profiles of wild strain and cured derivatives of *Rhizobium sp.*DC-2 : Lanes 1 and 8- *Agrobacterium tumifaciens* C58; lanes 2, 3 and 7 - wild strain; lanes 4,5 and 6 - plasmid cured isolates: PLR1, PLR 2 and PLR3; lanes 9, 10 and 11 *Rhizobium galegae* strains HAMBI 1141, HAMBI 1146 and NBIMCC 2250(BG7).

Table 1. Growth and mineral nutrient contents of wild strain and cured derivatives of *Rh. sp* DC-2 under Cu stress

Treatment	Cells Dwt g/l		Cu, µg/g Dwt		Mg, mg/g Dwt		Ca, mg/g Dwt		K, mg/g Dwt	
	1**	2	1	2	1	2	1	2	1	2
Cont	0.54	0.59	0.12	0.11	0.67	1.59	0.54	0.50	6.49	5.20
200	0.49	0.59	0.68	1.35	0.20	1.29	0.65	0.63	7.00	6.30
300	0.47	0.58	0.73	1.70	0.11	1.38	0.72	0.98	2.20	3.70
400*	0.20	0.46	0.10	2.20	0.04	1.64	0.44	1.04	1.70	2.10

* -µmol Cu, ** - 1- wild strain, 2 - mean value of the isolated cured derivatives

Correspondingly, the rhizobial derivatives have found to accumulate more Cu, Ca and Mg but less K when grown on the toxic Cu concentrations. The increased content of bounding Cu was found to the excreted in the medium extracellular polysaccharides by rhizobial cells under the stress treatments. These changes were corresponded to the alterations in the soluble protein patterns of the plasmid-cured derivatives. At the same time, the 200 ppm Cu treatment had not changed the nodulation rate of black locust plants inoculated with the wild strain, although the ARA rate and ureide content of nodules were decreased (Table 2).

Table 2. Nodulation and N_2 fixation of 200 ppm Cu stressed *Robinia* nodules

Treat-ments	Nodules mgDwt/ nodule	ARA µmol/g Fwt/h	Ureides nmol/g Fwt	Nutrient contents of : Cu µg/g	Mg mg/g	Ca mg/g	N mg/g
				Dry weight			
Control	13.8	1.95	730	4.58	5.4	9.2	42.4
200ppm Cu	15.3	1.45	572	6.00	5.9	9.9	62.8

These alterations were accompanied by the increased accumulation of Ca, Mg and N in the stressed nodules. Comparing the data of Cu stressed free-living bacteria and *Robinia* nodules, the involvement of the rhizobial plasmid DNA in the determination of the physiological Cu stress tolerance of free-living and nodulating *Rhizobium sp.* DC-2 strain is discussed.

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BIOREMEDIATION OF WATERS CONTAMINATED WITH HEAVY METALS BY A PASSIVE SYSTEM

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Mine drainage waters contaminated with toxic heavy metals (copper, zinc, cadmium) and sulphates were treated by a passive system consistng of an anoxic alkalinity-producing and sulphate-reducing cell.

The cell was a plastic column filled with a mixture of solid biodegradable substrates (spent mushroom compost, sawdust, cow manure) and was polluted by a community of different metabolically interdependent microorganisms.The sulphate-reducing bacteria were the prevalent microorganisms in this community. The contaminated waters were treated under both batch and continuous-flow conditions. The removal of metals in the cell was very efficient and their concentrations in the cell effluents were lower than the relevant permissible levels for waters intended for use in the agriculture and/or industry.

The microbial dissimilatory sulphate reduction and the sorption of contaminants on the organic matter in the cell were the main processes connected with this removal. However, some other processes such as biosorption, bioaccumulation and bioagglomeration of the metals by the microbial biomass were also involved in the water clean-up.

P 8

MICROBIAL COMMUNITIES IN AN URANIUM ORE DEPOSIT SUBJECTED TO IN SITU LEACHING

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The microflora of an uranium ore deposit subjected to in situ leaching was studded to establish the role played by the different microorganisms in the solubilization of uranium. Mixed populations of the acidophilic chemolithotrophic bacteria *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* were used in bioreactors with enhanced aeration to oxidize the ferrous ions to ferric state in diluted sulphuric acid solutions (with a pH of about 1.7)
The acidified ferric sulphate leach solutions obtained in this way were injected underground through a large number of boreholes to reach the ore body and to oxidize the tetravalent uranium to the hexavalent state. This oxidation was connected with the reduction of ferric ions to the bivalent state. The pregnant solutions containing dissolved uranium and ferrous ions reached the ground surface through productive boreholes and were treated to remove the uranium by ion exchange and to regenerate the ferric ions in the above-mentioned reactors. It was found that a microbial community consisting of the above-mentioned iron-oxidizing chemolithotrophs was establish in the aerobic zones located around the bottom ends of the injection boreholes. These chemolithotrophs were characterized by a well expressed ability to oxidize the U^{4+} directly and to use this reaction as a source of energy, including in the process of CO_2 assimilation. These bacteria were also able to oxidize the U^{4+} under anaerobic conditions, using the Fe^{3+} ions as electron acceptors. The anaerobic oxidation was not connected with an assimilation of CO_2.
The chemolithotrophs in the deposit were further characterized by their ability to grow in the presence of high uranium concentrations and a very low pH values (lower than 1.0) and by their very high iron-oxidizing activity.

REMOVAL OF HEAVY METALS FROM URANIUM MINE DRAINAGE USING SULFATE-REDUCING BACTERIA

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The presence of high concentrations of sulfate and metals in mining water, including radionuclides is one of the major problems connected to the closure of uranium mines. Large volumes of acidic water from the mines and dumps collect in lakes. The water cannot be disposed off until it has been treated in some way as it poses a direct threat to drinking water and agriculture. Treatment of this water using conventional methods is very expensive, therefore it is desirable to find an alternative method which is very effective and low in costs.
Decontamination of this water requires a number of steps: sulfates have to be removed, (heavy) metals and radionuclides have to be eliminated and collected and the water has to be neutralized.

A microbiological approach in which sulfate-reducing bacteria are used is a feasible solution: reduction of sulfate results in the precipitation of metal sulfides and neutralization of the water. Metals such as aluminium which don't precipitate as sulfides but stay in solution can be removed by biosorption. Uranium can be reduced from the soluble form to the insoluble form thus eliminating it from the water. Costs for this process result largely from the need for a carbon and energy source for the bacterial metabolism. It is therefore important to use a cheap electron donor which serves at the same time as a carbon source: methanol and whey are such substrates. They are both inexpensive and widely available.
A number of sulfate-reducing strains have been isolated which are suitable for this bioremediation process as they fulfill one or more of the following selection criteria: they are metaltolerant, acidotolerant, reduce uranium and can be used in biosorption processes and they utilize methanol and whey as sole sources of carbon and energy. Data from lab scale experiments and preliminary pilot scale tests are being presented.

P 10

MICROBIAL INVESTIGATIONS OF URANIUM NATURAL ANALOGUES

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Many countries have proposed concepts for deep subsurface disposal of nuclear fuel waste. These concepts are subject to rigorous safety assessment. Since laboratory experiments can only be conducted over relatively short time scales, studies of natural analogues that have existed over geological time scales can help in modelling and safety assessment. Two natural analogues consisting of uranium deposits have been investigated for their microbial populations. The Oklo region of Gabon, Africa, is the site of several uranium deposits which acted as natural nuclear reactors about 2 billion years ago. The uranium and associated fission products, as a natural analogue for nuclear fuel waste, have remained in place since that time, even though many of the reactors are now close to the surface and oxygenated groundwater recharges rapidly through the sites. Samples were collected from the Bangombé site and analysed for redox controlling microorganisms, sulfate reducing bacteria (SRB), iron reducing bacteria (IRB) and aerobic heterotrophs. It was shown that IRB and aerobic heterotrophs predominate in keeping the reactor zone reduced. A second uranium deposit was studied in Palmottu, Finland, this time in hard rock, mainly gneiss. Palmottu is a closer analogue to Swedish, Finnish and Canadian nuclear waste disposal concepts that propose disposal in hard shield rock. Both oxidized and reduced groundwater samples from Palmottu were investigated for SRB and IRB, while reduced waters were investigated for a wider range of anaerobes, including acetogens and methanogens. The natural populations of these sites show a broad diversity of redox controlling microorganisms. Many IRB and SRB are also capable of reducing uranium, with U(VI) as the sole electron acceptor or in combination with another electron acceptor. The IRB and SRB in the Bangombé and Palmottu sites may also contribute to uranium reduction.

ELECTRO-OPTICAL STUDY OF THE BACTERIAL SUSPENSIONS AT BACTERIAL – METAL INTERACTION

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The microbial cells of some strains metabolize toxic compounds with help special enzyme systems of the preliminary metabolism. These processes conduce to the redistribution of the charges in the microbial cells and may be register by few biophysical methods such as cell electrophoresis, impedance and electro-optical (EO) methods. We used in our work EO method. EO analysis is based on the recording of changes in optical characteristics of cell suspensions under the orienting effect of an electric field.

Previously, we investigated the EO properties and specific respiratory activity of cells of *Brevibacterium sp.* strain 13PA, which possess an inducible enzyme, amidase, and are able to utilise acrylamide as the sole carbon source. We demonstrated the selectivity of changes in the cell EO properties during acrylamide metabolism and found that the most dramatic changes took place at the first five frequencies of an orienting field (10-1000 kHz) [1,2]. We speculated that similar alteration in the EO properties of micro-organisms could also be typical for other strains possessing enzyme systems for preliminary metabolism of toxic compounds. To test this hypothesis, we used cells of *Acinetobacter calcoaceticum* strain A-122 possessing an inducible enzyme system for preliminary metabolism of *p*-nitrophenol. After optimisation of growth conditions, we found that *p*-nitrophenol (0,1mM to 1,0 mM) preincubation of *A. calcoaceticum* A-122 cells resulted in alteration in the EO properties of a microbial suspension at frequencies ranging from 10 to 502 kHz with the p-nitrophenol concentration dependence of the EO effect being near-linear over the range 0,1 mM to 0,8 mM [3]. Also studied was the respiration activity

of the A-122 cells towards various *p*-nitrophenol concentration. The concentration dependence of the specific respiration activity was also linear over the range 0,11 mM to 1,0 mM. The data generated from these studies indicate that the change in the EO properties observed in *A. calcoaceticum* A-122 cells is associated with the activity of the enzyme system for the preliminary metabolism of *p*-nitrophenol. In sum, the present finding confirm the relationship between the changes in the cell EO properties and the enzymatic processes occurring in microbial cells.

We investigated influence of the some metals (copper, zinc, nickel, and cobalt) on the degradation activity of the microbial cells. Since degradation activity of the cells depends from the activity of the enzyme systems we estimated the degradation activity with help EO analysis of the microbial suspension and specific respiratory activity of the cells. Accordingly EO analysis may be the additional tool for estimation of the degradation activity of the microbial cells at the action of the metals.

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THE STUDY OF REDUCTION OF HEPTAVALENT TECHNETIUM BY ACIDOPHILIC THIONIC BACTERIA

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The most attention in this study was paid to *Thiobacillus ferrooxidans*. It is known that this bacterium has different ability for recovering of energy. Brock and Gustavson [1] had found that *T.ferrooxidans* capable to grow anaerobically on sulfur reducing trivalent iron.

The aim of this study was to investigate the ability of *T.ferrooxidans* and *T.thiooxidans* to use heptavalent technetium as electron acceptor.

We dealt with technetium-99 (half-life is $2.12*10^5$ y, specific activity - 1.7 mCi/ mmol). Radioactivity of solutions were determined by scintillation counter after removal of cells by centrifugation. The experiments were carried out in 9K media [2] containing 5 g/L ferrous iron and 50 mg Tc/L under aerobic and anaerobic conditions. *T.ferrooxidans* can grow till concentration of technetium 100 mg/L. *T.ferrooxidans* proved much more resistant to technetium than purple nonsulfur bacteria, which are sensitive to 1 mg Tc/ L, but less resistant than green algae, which can grow at 600 mg Tc /L. [3] After 5-7 days of cultivation the radioactivity of solution was decreased average by 35%. During bacterial developing in this media colloidal particles were formed. The colloid was freezed out in freezing camera. It contained technetium and iron. Speciation of Tc was carried out by paper chromatography and scintillation counting. Computer-assisted processing of chromatogram showed that only 11% of the technetium was heptavalent here, 61.6% was transformed into a pentavalent state, and 27.5% was transformed into tetravalent state. The ratio of valent form was not constant. When *T.ferrooxidans* was grown anaerobically on molecular sulfur and pertechnetate 60% decrease of radioactivity was observed and only heptavalent and tetravalent technetium were found here. After growth of *T.ferrooxidans* on pyrite ore and pertechnetate radioactivity of solution was decreased by 40%.

T.thiooxidans also can grow anaerobically using sulfur and pertechnetate. Ferric iron was added before the end of experiment for precipitation of reduced technetium. The radioactivity of solution was decreased by 70% in average.

Therefore *T.ferrooxidans* can oxidize ferrous salts anaerobically using pertechnetate as electron acceptor. Pertechnetate can served as electron acceptor for *T.thiooxidans* too. Practical application of the results obtained for the removal of heptavalent technetium from radioactive solutions is suggested.

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MOLECULAR AND RADIOCHEMICAL ANALYSIS OF *Thiobacillus ferrooxidans* STRAINS RECOVERED FROM A URANIUM WASTE PILE IN SAXONY

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In natural bioleaching systems the autochtonic population of microorganisms is involved in the solubilization of metals from solid minerals by direct and/or indirect metabolic activities. The widest spread mesophilic group of bioleaching microorganisms are the chemolithotrophic bacteria *Thiobacillus ferrooxidans, Thiobacillus thiooxidans,* and *Leptospirillum ferrooxidans* /1/, /3/. *Thiobacillus*-strains recovered from a former uranium mine in Saxony, Germany were characterized using pulsed field gel electrophoresis (PFGE) and repetitive primer amplified polymorphic DNA (rep-APD) fingerprinting. Six novel isolated *Thiobacillus*-isolates were categorized as *Thiobacillus ferrooxidans.*

The PFGE fingerprints of the waste isolates were sample-specific. The sites of sampling have different depths and metal compositions. Three of the isolates, TFSS1, TFSS2, and TFSS6 were recovered from a sample drawn from a depth about one meter under the surface, where the concentration of uranium was low. The three other samples TFSS3, TFSS4, TFSS5 were recovered from a depth between two and three meters. The concentration of uranium at that site was estimated to be three times higher than those in the other sample mentioned above.

The six novel *Thiobacillus ferrooxidans* isolates from a particular rep-APD group are closely related to the strain *Thiobacillus ferrooxidans* ATCC 33020, also recovered from an uranium mine in Japan.

Furthermore it is known that *Thiobacillus ferrooxidans* strains can accumulate uranium. This ability is interesting for bioremediation of uranium in contaminated soils/2/. For accumulation studies we incubated three *Thiobacillus ferrooxidans* strains (ATCC19859, ATCC23270, ATCC33020) from the American type culture collection and *Thiomonas cuprina* DSM 5495 from the Deutsche Sammlung von Mikroorganismen und Zellkulturen with uranium (VI). The results have shown that the bacterial isolates (*Thiobacillus ferrooxidans* ATCC33020 and *Thiomonas cuprina* DSM 5495) both recovered from an uranium waste pile possess a higher capability to accumulate uranium(VI) than ATCC19859, ATCC23270. For desorption studies we extracted the biomass with 0.001M EDTA/0.01M TRIS solution. The desorption studies indicate that the main part of the uranium is strongly fixed onto the biomass of the strain ATCC 33020, recovered from an uranium waste pile.

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P 1

INVESTIGATION OF BACTERIAL DIVERSITY IN A SOIL SAMPLE OF A DEPLETED URANIUM MINING AREA NEARBY JOHANNGEORGENSTADT, SAXONIA, VIA 16S-RDNA-SEQUENCE ANALYSIS

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We report results obtained for the analysis of the bacterial diversity of a soil sample isolated in a pseudo-sterile manner from four-to-five meter depth at so called "site A" of a former Saxonian uranium mining area nearby Johanngeorgenstadt, Saxony, Germany ("Haberland uranium mining waste pile") correlated with a relative acidic (pH 4.5) surface water sample showing an elevated uranium concentration (58 mg/L). We applied the 16S-rDNA PCR amplicon clone library sequencing method. The pCRII-TOPO-vector was used for cloning and 100 randomly selected soil bacteria 16S-969-1406-rDNA clone sequences were compared against GenBank and RDPII (Ribosomal Database Project II) database reference sequences using 'Blast 2.0' and 'RDPII Sequence Match 2.7' respectively. Through RDPII- 'Chimera_Check' detection nine apparently chimeric sequences were excluded from the analyses. For phylogenetic comparisons representatives of the bacterial domain were chosen from the RDPII-"Small Subunit rRNA: Representative Prokaryotic Listing (July 31, 1998)". Additional reference sequences were chosen on grounds of the results gained via the comparisons against the sequence databases. The bacterial affiliations obtained by each of the two database comparisons are mainly identical to the phylogenetic analysis results. Three major taxa are highly represented in the soil sample 16S-rDNA library: Purple bacteria (24 %); Green-non-sulfur bacteria (41 %) and bacteria of the Fibrobacter, Acidobacterium subdivision (19 %). Only 5.5% of the clones belong to the Gram-positive phylum. Eight percent of the clones cluster with the DA052-clone [1], which probably represents a new lineage in the domain Bacteria. Solely one clone each was found for the Fusobacterium and relatives division and for the Paraphyletic assemblage, Leptospirillum/ Nitrospira subdivision respectively. The majority of the Purple bacteria belongs to the γ-Subdivision (15.4 %), while 4.4 % each are associated with the α- and β-subdivision. Within the Gram-positive phylum clones three of the five clones are High-G+C-subdivision members. Nevertheless the phylogenetic affiliation of nine of the aforementioned clones remains uncertain.

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P 18

BIOSORPTION OF HEAVY METALS BY *Myxococcus xanthus:* CELLULAR LOCALIZATION

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The ability of microorganisms to remove metals from solution is well documented (see, for example [1]. Both living and dead biomass is capable of metal accumulation. Effluents from many industries (mining, electroplating, etc.) contain metals in excess of permitted levels. Removal of such metals by waste microbial biomass may be economically feasible.

The present study deals with uranium, lead, silver and lanthanium biosorption by *M. xanthus* biomass in wich dry biomass is demonstrated to be a more efficient biosorbent than wet biomass, accumulating up to 0.99 mmol of uranium, 0.97 mmol of lead, 0.19 mmol of lead and 0.99 mmol of lanthanium per gram of biomass. Electron microscopy and X-ray spectroscopy were used to determine location of uranium, lead, silver and lanthanium biosorbed by *M. xantus* cells. The biosorbed uranium and lead were located in the cell wall cell and within the characteristic extracellular polysaccharide of this microorganism, while the silver and lanthanium (Fig.1) were located in the cell wall, inside of the cell and within the extracellular polisaccharide.

Fig.1. Location of lanthanium biosorbed by wet biomass of *M. xanthus.* Transmission electron micrographs of the tin section of the wet biomass. The lanthanium is fixed fundamentally, within the mucopolysaccharide (a) and on the cell wall (b).

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BACTERIA FROM URANIUM MINING WASTE PILE: INTERACTION WITH U(VI)

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Bacteria in soil, sediment, and water can have a significant influence on the transport of radionuclides and other heavy metals in nature. Certain bacterial strains can bioaccumulate large amounts of uranium which can be transported and released elsewhere in the environment /1-4/. These organisms may be applicable for bioremediation of uranium-contaminated soils such as uranium mining waste piles.

In uranium deposits a number of acidophilic chemolithoautotrophic bacteria have been identified which are able to oxidize sulphide minerals, elemental sulfur, ferrous iron, and in presence of uranium minerals also U(IV). Especially one representative of this group, *Thiobacillus ferrooxidans,* is of particular interest. The mechanism of the uranium oxidation was extensively studied, but only little information is available on the quantity, binding strength, reversibility, and structure of the bacterial complexes formed with U(VI) /5/. We studied the bioaccumulation of U(VI) by several strains of *Thiobacillus ferrooxidans* drawn from environments with different mineral compositions. Strains *T. ferrooxidans* ATCC 23270^T and ATCC 21834 were recovered from two different coal mines in USA and Japan, *T. ferrooxidans* ATCC 19859 from a Canadian copper mine, and *T. ferrooxidans* ATCC 33020 from a uranium mine in Japan. We compared the results obtained with the *T. ferrooxidans* strains to those obtained with another acidophilic strain, *Thiomonas cuprina* DSM 5495^T, which does not belong to the genus *Thiobacillus* but was recovered from a German uranium mine. Sorption studies have shown that the origin of the strains has a significant influence on their capability to accumulate uranium. The amount of uranium bound to the biomass increases in the order *Thiobacillus ferrooxidans* ATCC 19859 (copper mine), ATCC 23270^T (coal mine) and ATCC 33020 (uranium mine) whereas *Thiomonas cuprina* shows the highest uranium uptake. In order to get information on the binding strength and the reversibility, we tried to remove the accumulated uranium from the biomass by different extractants. Extraction studies with sulfuric acid released none, and with EDTA only a small fraction of the U(VI) that was accumulated by the bacteria. These results show that the main part of the uranium forms strong complexes with the bacteria and cannot be removed by extraction. *Thiomonas cuprina* has different surface properties which leads to a different sorption and/or complexation behaviour towards uranium. The total amount of accumulated U(VI) for this strain is higher than for the *Thiobacillus ferrooxidans* strains, but the binding is weaker. A larger amount of the uranium could be released from the biomass by EDTA-treatment. For characterization of the formed bacterial-UO_2^{2+}-complexes, time-resolved laser fluorescence spectroscopy (TRLFS) was used. The interaction with the biomass causes a strong bathochrome shift of the emission bands and an increase of life time by a factor of 1.8 for *Thiobacillus ferrooxidans* ATCC 19859, 2.0 for *Thiobacillus ferrooxidans* ATCC 21834, 2.5 for *Thiobacillus ferrooxidans* ATCC 33020, and 1.5 for *Thiomonas cuprina* DSM 5495^T. This confirms the formation of strong inner-sphere complexes for all strains examined. In the case of *Thiobacilli* the red shift of the emission bands and the fluorescence life time of the bacterial complexes increase in

the same order as the capability of the strains to accumulate uranium. Given that these changes of spectroscopic parameters are a consequence of the influence of the ligands on the coordination sphere of the uranyl ion, we can draw the following conclusions concerning the binding strength of the complexes:

- Comparing the *Thiobacillus ferrooxidans* strains, the isolate from the copper mine (*Thiobacillus ferrooxidans* ATCC 19859) forms the weakest, the strain from the uranium mine (*Thiobacillus ferrooxidans* ATCC 33020) the most stable complexes with UO_2^{2+}.
- *Thiomonas cuprina* shows a higher accumulation (relatively to the dry weight of the biomass) than the *Thiobacillus ferrooxidans* strains, but forms less stable complexes with U(VI).

These results are in good agreement with the desorption studies.

Further investigations were performed with *Bacilli*, another important group of bacteria wildly distributed in a large variety of natural habitats. We studied the interaction of vegetative cells and spores of three *Bacillus* isolates (JG-A 30, JG-A 12, JG-A 22, classified as *Bacillus cereus, Bacillus sphaericus, Bacillus megaterium,* respectively) from a uranium mining waste pile (Haberlandhalde, Johanngeorgenstadt) in Saxony, Germany, and the corresponding reference strains of these species with U(VI). The sorption studies have shown, that *Bacilli* can accumulate high amounts of uranium. In the concentration range examined (11 – 214 mg/L), the uranium is taken up linearly with increasing initial uranium concentration by the isolate A 22 and its reference *B. megaterium* strain (vegetative cells and spores). In the cases of the other strains, the amount of uranium bound to the biomass approaches a limiting value for higher uranium concentrations (> 80 mg/L). Comparing the sorption behaviour of vegetative cells and the spores, for A 30 and *Bacillus cereus* no significant differences could be observed. In the case of A12/ *Bacillus sphaericus*, the spores show a higher uranium accumulation related to the dry weight of the biomass. For *Bacillus megaterium*, sorption properties of the vegetative cells and the spores are almost identical whereas in the case of the corresponding isolate the capability to accumulate uranium increases with sporulation. Contrary to the results of the *Thiobacilli*, the uranium bound to the vegetative cells was released almost quantitatively by EDTA-extraction. In agreement with the similar sorption behaviour of the vegetative cells and the spores of the pair A 30 / *Bacillus cereus*, also comparable amounts of uranium were extracted from these spores. For the other strains, the part of the non-extractable uranium on the biomass increases with the formation of the spores. Especially in the case of A 12 / *Bacillus sphaericus*, strains forming very small circular spores, the fraction of extractable uranium was smaller than 40% / 50 %. The characterization of the formed bacterial-UO_2^{2+}-complexes by time-resolved laser fluorescence spectroscopy has proved the formation of inner-sphere complexes with the biomass for all strains examined. In accordance to the results of the *Thiobacilli*, the fluorescence spectra of the bacterial complexes show a strong red shift compared to the hydrated uranyl ion, and the life time of the fluorescence emission increases.

In addition, we studied the reduction of U(VI) by sulfate-reducing bacteria, *Desulfovibrio desulfuricans* DSM 642^T from a soil near a gas main, and a natural *Desulfovibrio* isolate (JG 1) recovered from a uranium mining waste pile (Haberlandhalde, Johanngeorgenstadt, Saxony, Germany). The representatives of the genus *Desulfovibrio* can use U(VI) as a terminal electron acceptor to obtain energy

for growth whereas U(IV) precipitate is formed /6,7/. In order to compare the reduction capacity of the natural isolate and the reference strain, we studied the conversion of U(VI) to insoluble U(IV) as a function of time for different pH values. For *Desulfovibrio desulfuricans*, the main transformation of U(VI) occured during the first 24 hours. From 24 to 100 hours only a slight increase of the U(IV) amount was observed. After 100 hours, limiting values were reached:

pH 3.2:	41.2 ± 5.0 $mg/g_{dry\ weight}$
pH 4.2:	81.1 ± 6.9 $mg/g_{dry\ weight}$
pH 5.0:	198.5 ± 9.9 $mg/g_{dry\ weight}$
pH 6.1:	316.2 ± 0.1 $mg/g_{dry\ weight}$

The yield increased from 10.3 to 99.2 % when the pH was changed from 3.2 to 6.1, respectively. The increase of the rate and yield with increasing pH corresponds well to the neutral pH-optimum for survival of this reference strain. For JG 1 a quantitative reduction was observed within 15 minutes for all pH-values examined. These results show that in the case of the natural isolate microbial reduction is much more effective and less dependent on the pH. While the reduction capacity of *D. desulfuricans* already decreases significantly at pH 4.2, in case of JG 1 the yield even at pH 2.8 is similar to that in neutral pH-region. Therefore, JG 1 seems to be more inert to chemical changes of the surrounding environment and can use U(VI) instead of sulfate for obtaining energy more efficiently than the reference strain, what is of particular interest for bioremediation purpose.

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SELECTIVE ACCUMULATION OF HEAVY METALS IN DRAIN WATERS OF A URANIUM MINING WASTE PILE BY INDIGENOUS *BACILLI*

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The interaction was studied of three *Bacillus* isolates, recovered from a uranium mining waste pile in Saxony, Germany, with different heavy metal ions present in the original ground water of the pile.

Using 16S ARDRA and RAPD, the three isolates were classified as *B. cereus, B. megaterium* and *B. sphaericus*. The ability was investigated of vegetative cells and spores of the indigenous *Bacillus* strains as well as those of the corresponding reference strains *B. cereus* ATCC 4415, *B. megaterium* NRRL B5385, and *B. sphaericus* NCTC 9602 to accumulate Al, Ba, Cd, Co, Cr, Cs, Cu, Fe, Ga, Mn, Ni, Rb, Pb, Si, Sn, Sr, Ti, U, and Zn. It was clearly shown that all strains studied accumulated selectively large amounts of U, Pb, Cd, Cu, and Al.

Co, Mn, Ni, Zn, and Ga were only weakly accumulated. The binding of Ba, Ga, Mn, Ni, and Zn was species- and in some cases even strain-specific.

A SIMPLE METHOD FOR DETERMINATION OF THE ABILITY OF SULPHATE REDUCING BACTERIA TO REDUCE U(VI)

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A gram-negative sulphate reducing bacterium of the genus *Desulfovibrio*, named strain UFZ B490 (former JG1), was isolated from the uranium dump „Haberland" near Johann-Georgenstadt in the south-east of Germany.
A simple agar plate screening method was used to determine the ability of the isolated strain to reduce hexavalent uranium to the tetravalent state, named uraninite. The test is based on the visualisation of the metal reduction by the cells of the colonies without addition of reagents.
The screening method for the isolation of metal-accumulating microorganisms of PÜMPEL & PERNFUß et al. (1995) was used as the basis of this test.
Agar plates with a bicarbonate buffer medium for sulphate reducing bacteria were inoculated with the isolated *Desulfovibrio* UFZ B490. The well-developed colonies were covered with a top agar, which contained U(VI). After a incubation time of approximately 7 days the result were obtained.
The colour colonies had been changed from colourless to black. Under a stereo microscope the dark precipitates of the formed uraninite on and around the colonies can be seen clearly.

The studies of PANAK & HARD et al. (1998) presented the ability of the isolate UFZ B490 (JG1) to reduce the U(VI) very rapid in comparison to the tested reference strain *Desulfovibrio desulfuricans* (DSMZ 642^T).
According to the literature (LOVLEY & PHILLIPS, 1992 / LOVLEY & RODEN et al., 1993) sulphate reducing bacteria don't grow by reducing U(VI), the sole electron acceptor. The results obtained in our studies using the isolated strain UFZ B490 for a U(VI) reduction didn't confirm these findings.
Figure 1 presents the result of an experiment which tested the reduction of hexavalent uranium in 30 mM bicarbonate buffer containing lactate as the electron donor, U(VI) as the electron acceptor, ammonia-nitrogen and traces of iron. Tests were carried out with and without the addition of cysteine.
Protein increase and U(VI) reduction show the increasing influence of cysteine on U(VI) reduction over the time. However reduction of hexavalent uranium occurred in the absence of cysteine, too.

Additional experiments were carried out which investigated the influence of various U(VI) concentrations (1 to 3 mM hexavalent uranium) on the growth of the isolated *Desulfovibrio* in presence of ethanol as the sole electron donor, without the addition of cysteine.
These experiments showed that U(VI) reduction and growth occurred with concentrations of 1 mM and 2 mM U(VI), but not 3 mM U(VI). In samples which contained 2 mM hexavalent uranium higher consumption of ethanol was observed compared to samples with 1 mM U(VI).

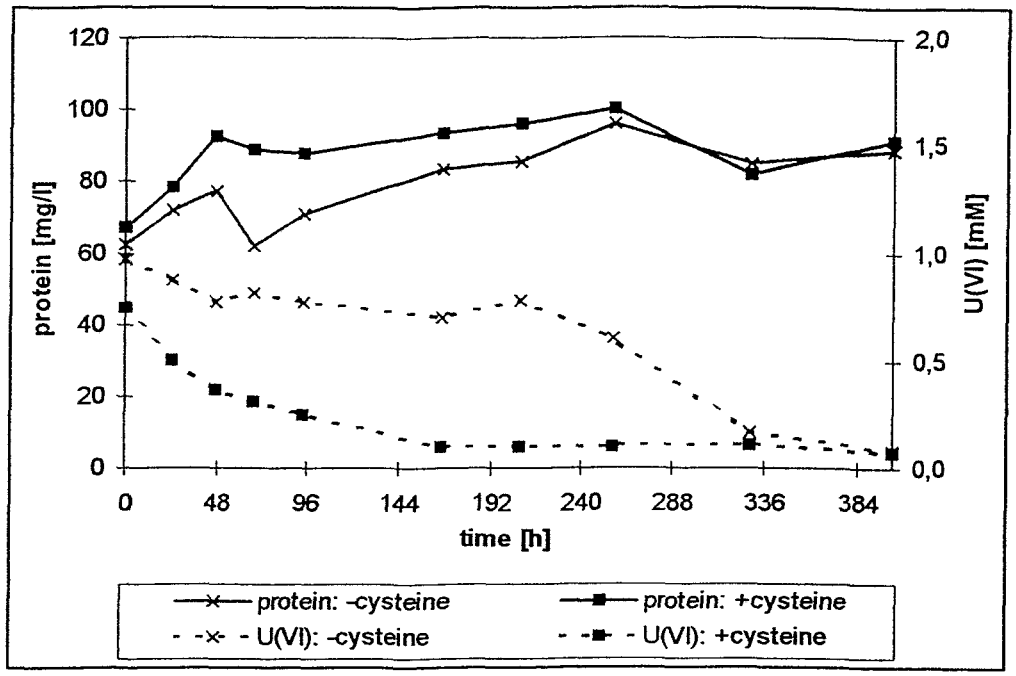

Fig.1: Growth and the U(VI) reduction by UFZ B490 in presence and absence of cysteine tested in a bicarbonate buffer medium containing only lactate as the electron donor, U(VI) as electron acceptor, ammonia-nitrogen and traces of iron

The preparation of balances for the utilisation of the electron donor (lactate) and the reduction of electron acceptors for sulphate and uranium reduction are under investigation at this present time. The aim of future studies will be the development of a metabolic scheme of U(VI) reduction by UFZ B490, which will be helpful in developing a bioremediation process.

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CHARACTERISATION OF THE SURFACE LAYER PROTEIN OF THE *BACILLUS SPHAERICUS* ISOLATE JG A-12 FROM A URANIUM WASTE PILE

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Many bacteria possess a crystalline protein or glycoprotein surface layer (S-layer) as the outermost component of their cell wall. The function of this protein lattice in different bacteria is not uniform and has been studied in detail only in some cases. The protein layer may work as a protective coat, molecular sieve, ion trap, may act as a framework or may be involved as a structure in cell adhesion and surface recognition /1, 2/. In addition, it was demonstrated that the isolated lattice interacts with metal ions by forming metal clusters /3/. The latter might be used for a development of biotechnological procedures to remediate heavy metal contaminated wastes. In this work a bacterial surface layer of a natural *Bacillus* isolate JG A-12, which was recovered from a uranium waste pile near the town Johanngeorgenstadt in Saxony, was analyzed and compared to the surface layer of a reference strain (*Bacillus sphaericus* NCTC 9602).

The natural isolate JG A-12 was phylogenetically affiliated to the species *Bacillus sphaericus* by the use of the RFLP analysis of the PCR-amplified 16S rDNA. The genomic macro-fingerprints of the two *B. sphaericus* strains obtained by pulsed-field gel electrophoresis were strain specific.

Bacillus sphaericus NCTC 9602 and the isolate JG A-12 were grown in batch culture until the end of their exponential growth, harvested by centrifugation and stored at -20 °C. To isolate the S-layer a modified method of Engelhardt /4/ was used: first the cells were disrupted with glass beads in a mixer mill. After that the pure S-layer sheets were produced by centrifugation of the crude extract and successive treatment of the pellet with Triton X-100 and lysozyme. The purity and the apparent molecular weight were examined with a denaturating polyacrylamid gel electrophoresis. The molecular weights of the S-layer proteins of both, the reference strain NCTC 9602 and the strain JG A-12, were estimated to be approximately 135 kDa. This size is about 10 kDa larger than the one, published for the S-layer protein of another *Bacillus sphaericus* strain 2362 /5/. The first 20 amino acids at the N-termini of the 135 kDa proteins of the strains 9602 and JG A-12 were identical. However, no similarity to the oblique (p2) *Bacillus sphaericus* 2362 S-layer protein was found /1, 5/. Interestingly, in the case of the uranium waste isolate, in addition to the S-layer protein a second, smaller protein with a size of 30 kDa was copurified. The amino acid sequence of the N-terminus of the 30 kDa protein was significantly different from those of the S-layer proteins. This small protein possesses a similarity to many flagellins of different bacteria. The highest similarity was found to flagella proteins from *Leptospira interrogans* (44 % identity and 70 % positives) and *Bacillus subtilis* (51 % identity and 70 % positives) as analyzed with BLASTP, databases PIR, and NR-AA. The lattice structures of the S-layers of the reference strain *Bacillus sphaericus* NCTC 9602 and of the uranium waste isolate JG A-12 were also characterized using transmission electron microscopy. Fig. 1 shows the isolated and negatively stained (with

uranyl acetate) tetragonally arranged S-layer (p4) from both *Bacillus sphaericus* strains. In (B) fragments of the bacterial flagellum (see the arrow) are indicated.

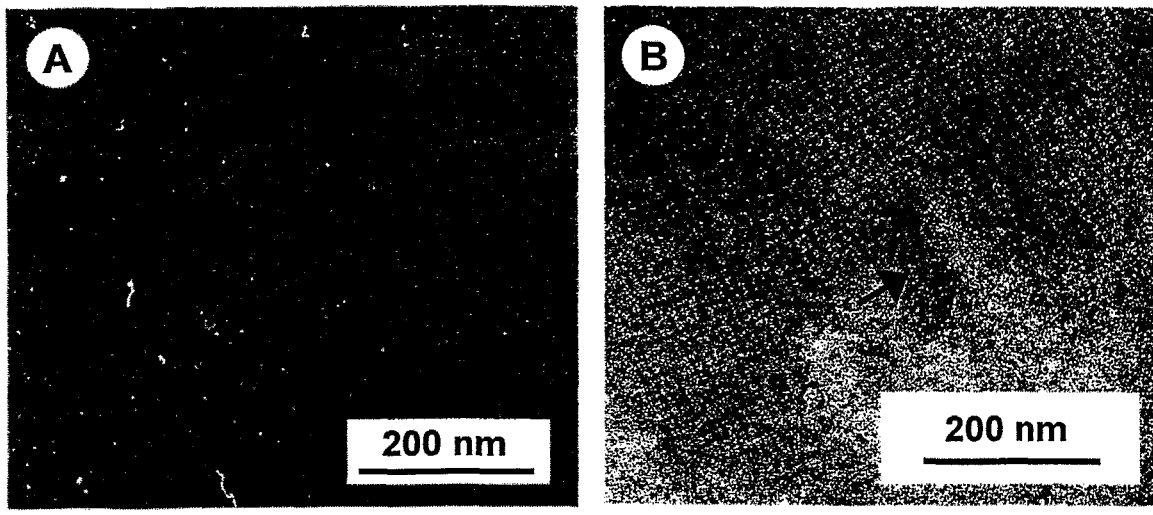

Fig. 1. S-layer of *Bacillus sphaericus* NCTC 9602 (A) and the *Bacillus sphaericus* isolate JG A-12 (B)

The isolate JG A-12 is the first *Bacillus sphaericus* strain recovered from a uranium contaminated environment and tested for the presence of S-layer. Surprisingly, both strains, *Bacillus sphaericus* 9602 and JG A-12, seem to possess the S-layer protein with the same structure. Proteolytical cleavage and sequencing of the internal protein fragments will be applied to prove this our first observation. Further studies of S-layers from other isolates (especially thiobacilli) recovered from the uranium waste pile „Haberland" are in progress.

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ACTINIDE COMPLEXATION BY FUNGAL HYDROXAMATE SIDEROPHORES

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The aim of the project is to identify fungal hydroxamate siderophores that are good actinide chelators. Hydroxamate siderophores are ligands secreted by micro-organisms under iron-limited conditions. They are very specific for Fe^{3+}, but can also bind well to other ions with similar charge/radius ratios, like the tetravalent actinides. First fungi are screened on dye agar plates to detect siderophores production and UO_2^{2+} complexation by siderophores. Both identified organisms and unknown fungi isolated from soil have been tested. Once suitable organisms have been found, they are grown in iron-limited liquid cultures. The number of siderophores produced and their isoelectric points are determined using thin layer chromatography and isoelectric focusing. These siderophores are then isolated and purified using chromatography techniques. The chelating abilities of the purified siderophores are investigated by determining the amounts of α-FeO(OH), $Th(OH)_4$ and UO_3 solubilized by them. The trace amounts of Fe^{3+}, Th^{4+} and UO_2^{2+} brought into solution are measured using inductively coupled plasma mass spectrometry.

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MICROCALORIMETRIC ACTIVITY TEST FOR BIOLEACHING

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Both, in biomining operations and for the uncontrolled bioleaching in mine waste heaps and tailings, there is the necessity to determine the activity of leaching bacteria for the evaluation of measures to control the bioleaching process. In several ecological investigations of leaching biotopes including calorimetric studies a correlation of the thermal power of samples with numbers of leaching bacteria was demonstrated [1]. Furthermore, the reaction energy of pyrite oxidation by pure cultures of leaching bacteria was determined calorimetrically with high accuracy [2]. Therefore, it might be possible to estimate the rate of bacterial metal mobilization in leaching biotopes by calorimetry. In further studies including laboratory experiments and investigations of leaching biotopes a calorimetric bioleaching activity test has been developed. The metal mobilization rate and the temperature dependence (energy of activation) of the bioleaching of different natural ores were determined in percolator experiments by calorimetry. In addition, the test was successfully applied to investigations of heavy metals containing wastes of the metal recovery industry (copper shale smelting plant in Helbra, Germany) where the leaching activity was dominated by the oxidation of PbS und ZnS. The oxidation of pyrite and marcasite was calorimetrically quantified for samples of waste heaps from german coal and lignite mining areas. The test enables the determination of both chemical and biotic leaching activity within 2 hours.

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MOLECULAR BACTERIAL DIVERSITY IN SOILS AND WATERS OF TWO EAST GERMAN URANIUM MINING WASTE PILES

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Bacterial diversity in two uranium waste piles near Johanngeorgenstadt and Gittersee/Coschütz, in Saxony, East Germany, was studied using several different approaches for direct molecular analysis.

The first approach was based on Ribosomal Intergenic Spacer Amplification (RISA) in total DNA recovered from soil and water samples from the wastes by direct lysis [1, 2]. The DNA patterns obtained by this method varied in dependence on the site and on the depths from which the samples were taken.

The second approach used, named repetitive primer amplified polymorphic DNA (rep-APD), is based on PCR amplification by the use of primers corresponding to the repetitive consensus sequences in bacterial genomes [3, 4]. This method is much more informative than RISA, due to the fact that it derives information from the whole bacterial genome and not only from a particular part of it as it is the case of RISA [5]. An example of this analysis using a BOX-primer is shown in Fig.1.

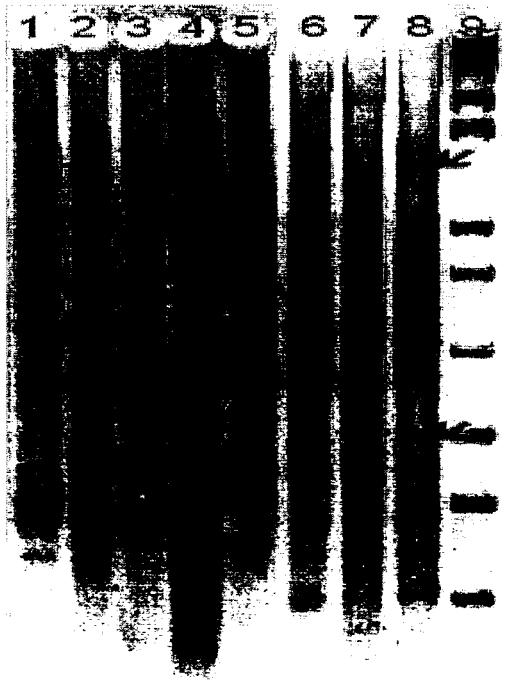

Fig.1: Box-rep-APD in total soil DNA recovered from Gittersee/Coschütz (Gitt.) and from „Haberland-Halde" Johanngeorgenstadt (JG)
1: Gitt.6, 2: Gitt.7, 3: Gitt.2, 4: JG-C 1-2, 5: JG-B 3-4, 6: JG-B 2-3, 7: JG-B 0-1, 8: JG-A 4-5, 9: 1kb plus ladder (Gibco BRL)

As seen in the figure the BOX-generated fingerprints are sample specific. However, for the samples drawn from the same site and depth they share significant similarity (compare Gitt. 2, Gitt. 6, Gitt. 7). Less similarity was found between the samples drawn from different depths (one to four meters under the surface) of the same site (compare JG-B 0-1, JG-B 2-3, and JG-B 3-4). Interestingly, common bands, demonstrating presence of common bacterial genomic structures were found in the BOX-patterns between many of the JG DNA samples (see the arrows in Fig.1). Moreover, in some cases similarity between the BOX-patterns of the samples from the two geographically different waste piles investigated was observed (see the double arrow in Fig. 1).

This result is consistent with those obtained by the use of another molecular approach, the 16S rDNA retrieval [6, 7] which was applied in our laboratory for more precise examination of the bacterial types present in the uranium wastes studied. The latter is based on a combination of RFLP and sequence analyses of 16S rDNA fragments amplified in the above mentioned soil and water DNA samples. Several hundred of the amplified 16S rDNA fragments were cloned in *Escherichia coli* and a large number of them was analysed (see Table 1).

Table 1. RFLP-typing of the 16S rDNA

Geographic origin	Number of:			
	Clones	Categorized	RFLP types	Common types
U-waste pile B 0-1 Johanngeorgenstadt	84	38	10	2
U-waste pile B 2-3 Johanngeorgenstadt	38	31	24	3
U-mill-tailing Gittersee/Coschütz	155	55	29	2

Many unique 16S RFLP patterns were obtained. However, several dominant RFLP types were shared by all soil waste samples studied. By the use of the sequence analysis it was demonstrated that these dominant types represent a β-subdivision of the Proteobacteria. The most dominant 16S rDNA group was affiliated to the species *Thiobacillus ferrooxidans*.

ACKNOWLEDGEMENTS

This work was supported by grant 7531.50-03FZR/607 from the Sächsisches Staatministerium für Wissenschaft und Kunst, Dresden, Germany. We thank Claudia Helling for providing us soil samples from the mill-tailing waste near Gittersee, Petra Panak for the water samples from the mill-tailing near Schlema, and Christoph Puers and Sabine Kutschke for the soil samples from the uranium waste pile near Johanngeorgenstadt

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STUDYING BACTERIAL TRANSFORMATIONS OF IRON IN A MODEL EXPERIMENT WITH KAOLIN.

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A stagnant moisture regime was modeled in kaolin from the Prosyanovskoe site. The wide range of ferric minerals contained in this kaolin makes it a very convenient object for studies of probable ways of bacterial transformations of iron in sedimentary environments. The basic minerals of the kaolin used are (per cent by weight) kaolinite 90, mica 1, illite 3-4, and quartz 5. Iron is contained in ferric minerals and in phyllosilicates. The ferric minerals are represented by hematite α-Fe_2O_3, magnetite Fe_3O_4, goethite α-FeOOH, and protolepidocrocite γ-FeOOH.

The experiment was performed in a 5-l glass reservoir 9/10 full of 60% kaolin suspension. All the openings in the reservoir were sealed with plugs equipped with outlets for sampling gaseous, liquid, and solid phases. Adding the culter medium with glucose (1g glucose/l suspension), an inoculum (5%), and running the experiment at 30°C compensated for the relatively short term of experiment (38 days). Inoculation was performed with the stable community of aerobic and anaerobic bacteria isolated earlier from the kaolin. The microbial community includes bacteria of following genera (in 10^5 cells/g): *Nitrobacter* (4), *Bacillus* (0.9), *Pseudomonas* (0.3), *Burkholderia* (0.1), *Nocardia* (0.4), *Caulobacter* (3), *Deinococcus* (4), *Arthrobacter* (0.7) *Clostridium* (4), *Bacteroides* (0.08) *Desulfovibrio* (1), *Desulfobacter* (0.3), a representative of metanogenic bacteria (0.05) and a representative of iron-reducing bacteria (2) [1].

For the bacterial enumeration, measures of microbial metabolites, pH and Eh during the development of the community common microbiological, analytical and physicochemical methods were used [2]. It is difficult to identify directly iron minerals in the kaolin because of their low content (0.7%). Hence, iron minerals were analysed after their concentration and subsequent isolation in 4 magnetic fractions characterized by the magnetic-field intensities H = 1, 4, 10, and 15 kOe. The composition of iron minerals was studied by transmission electron microscopy [3]. Sampling thermomagnetic analysis was used for studying the low-magnetic ferric oxides [4].

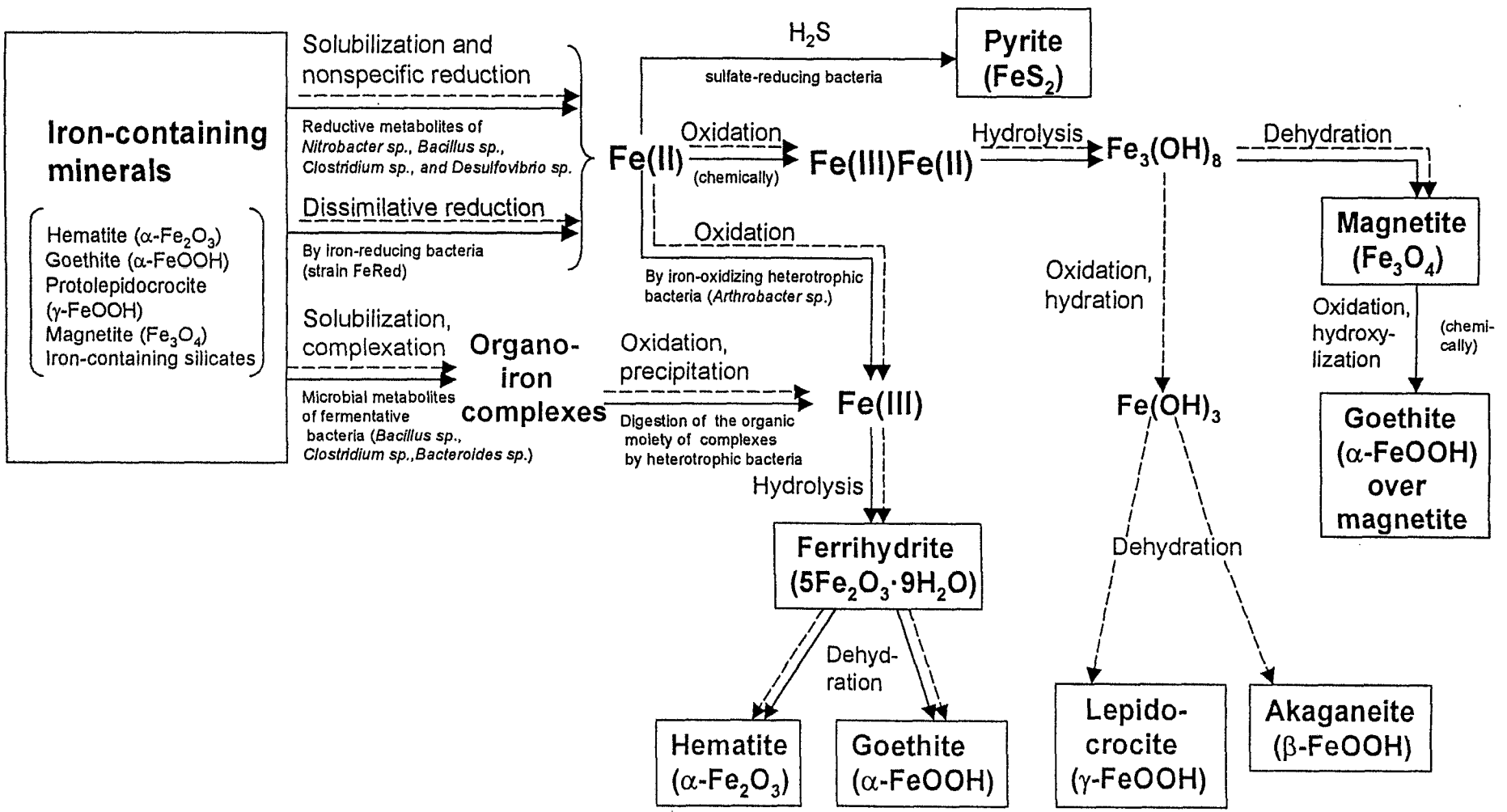

Fig.1. Microbial transformations of iron minerals in kaolin : (⟶) - under conditions of a stagnant water regime with some water exchange, and (⇢) - in conditions of a completely stagnant water regime.

Two stages were revealed in the development of microbial community. The first stage was characterised by active development of aerobic and facultatively anaerobic, and also fermentative bacteria. During this stage, glucose was consumed virtually completely, and organic acids (butyrate, acetate, lactate, and formiate) accumulated. The second stage was characterized by an increase in the number of anaerobic bacteria, by the production of propionic acid, and the release of CO_2, H_2, H_2S, and CH_4. By the end of the experiment, the stratification of the kaolin sediment into layer with different Eh and pH values was complete. The Eh value changed insignificantly and the pH value decreased (from 8.0 to 5.6) in the upper layer of kaolin under conditions of a stagnant water regime with some water exchange. On the contrary, the pH remained unchanged and the Eh decreased substantially (from 450 to -130 mV) in the lower layer, which formed in conditions of a completely stagnant water regime.

Investigation of the microbial community development and of the transformations of the iron minerals let us to create the model presented in Fig.1. The formation of thermodynamically unstable ferric hydroxides akaganeite β-FeOOH and lepidocrocite γ-FeOOH was noted only in conditions of a completely stagnant water regime. And formation of fine pyrite FeS_2 occurred only under conditions of a stagnant water regime with some water exchange. A decrease in chemogenic forms of goetite α-FeOOH and hematite α-Fe_2O_3 and an increase in biogenic forms of these minerals formed over ferrihydrite was established. An increase of the magnetite level may testify either to destruction of bonds between ferric minerals and phyllosilicates and better kaolin separation, or to magnetite formation. Magnetite in this system may form either purely chemically, with microorganisms participating indirectly, or through iron biomineralization performed by iron-reducing bacteria.

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BACTERIAL LEACHING OF METAL SULFIDES PROCEEDS BY TWO INDIRECT MECHANISMS VIA THIOSULFATE OR VIA POLYSULFIDES AND SULFUR

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Bacterial leaching of metal sulfides proceeds by two indirect mechanisms. *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* dissolve the acid-insoluble metal sulfides FeS_2, MoS_2, and WS_2 by the primary attack of iron(III) hexahydrate ions generating thiosulfate, which consecutively is oxidized to sulfuric acid. Almost all other metal sulfides, besides their susceptibility for iron(III) ions, are acid-soluble and, consequently may be dissolved by proton attack. The chemical oxidation of ZnS, $CuFeS_2$, PbS, MnS_2, As_2S_3, and As_4S_4 resulted in the formation of elemental sulfur via intermediary polysulfides. In the presence of *Thiobacillus ferrooxidans* as well as *Thiobacillus thiooxidans* the sulfur is oxidized to sulfuric acid. The acid (protons), in turn, dissolves the metal sulfide. This explains, why *Thiobacillus thiooxidans* is able to leach the latter metal sulfides.

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P 28 REMEDIATION OF HEAVY-METAL CONTAMINATED SEDIMENTS BY BIOLEACHING

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Heavy-metal contaminated sediments are a major environmental problem. The waters in Saxony alone contain about 18 million tons of sediments, 6 million tons of which have to be urgently removed. These sediments are in part anthropogenously polluted by heavy metals (mining, chemistry and processing industries), especially with Cd, Zn, Ni, Cu, Co, Mn, Pb and Cr [1].
When the dredged sludges contact air, the material turns acidic due to oxidation processes, the heavy metals become soluble and the sediments turn into an environmental risk. In view of the high costs and the deficient sustainability of landfill disposal there is a need for ecologically consistent remediation techniques. We are therefore developing a sediment treatment process based on the removal of heavy metals by bioleaching (Fig.1).

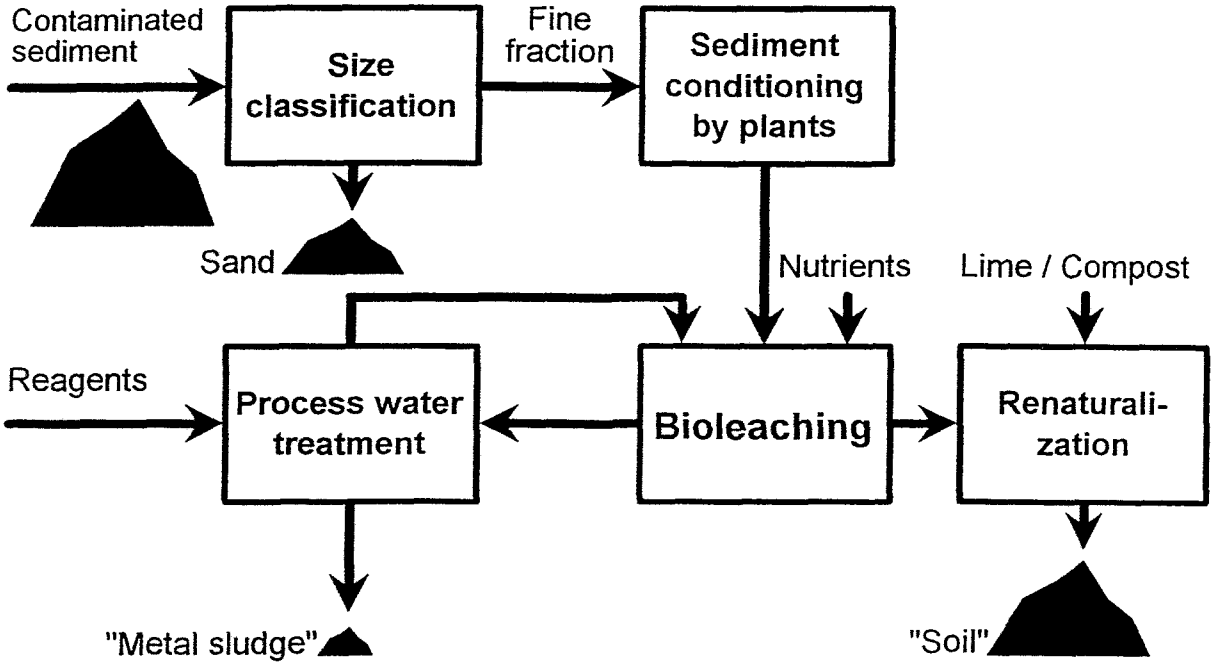

Fig.1. Scheme of the sediment treatment process

To accelerate the natural process of microbial leaching the indigenous *Thiobacilli* of the sediment are activated by elemental sulphur (S^0) addition and acidification. For economical reasons, only the solid-bed leaching (percolation principle) is applicable in large-scale processes [2].
Freshly dredged sediments are anoxic, practically impermeable to water and therefore unsuitable for treatment by the percolation principle. When dredged anoxic sludge is stored in the open, it gradually rots, is transferred into the oxic state and becomes permeable. To speed up the transfer into the oxic state, freshly dredged sludge was pretreated by being planted with various grasses such as *Phragmites australis*, *Agrostis stolonifera*, *Phalaris arundinacea* (Fig.2).

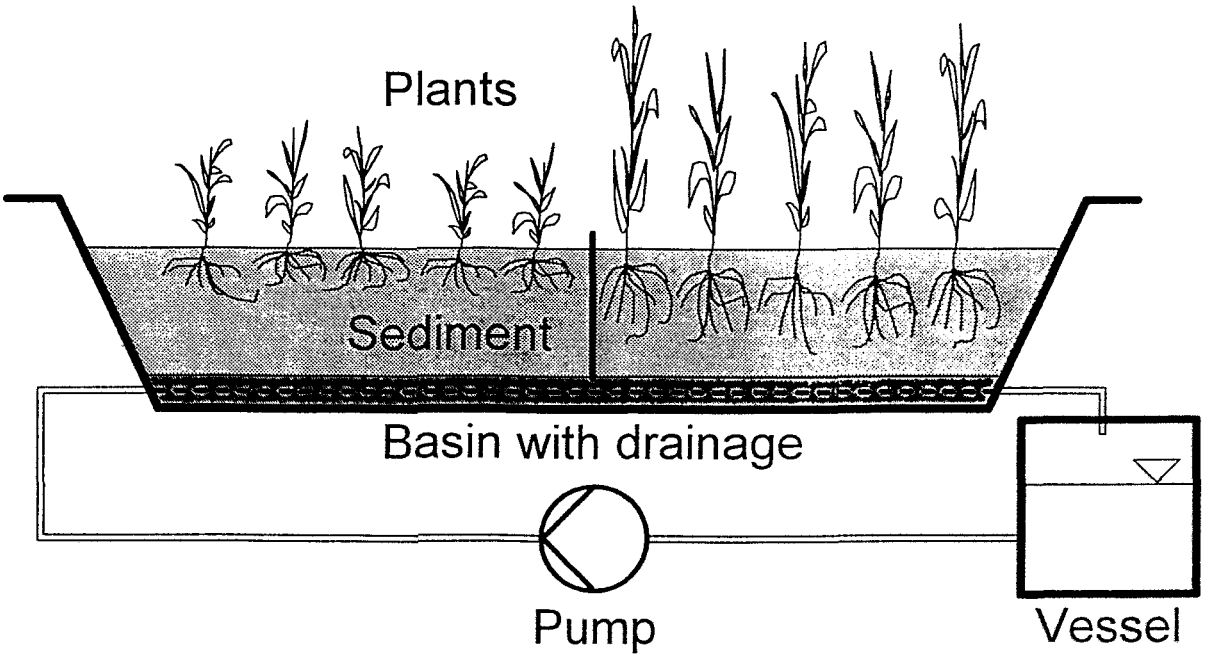

Fig.2. Scheme of the pilot-scale plant for conditioning anoxic river sediments

During rotting its water content and pH value decreased and the redox potential and content of sulphate increased. An enlargement of the pores and the average particle size during conditioning was also observed. After only 3 months the anoxic sediments had almost the same properties as oxic sediments which had been stored for over 6 years, and they were well permeable to water. The bioleaching of the conditioned sediments was comparable to the long-time stored sediments in both a laboratory percolator and a pilot plant (mean leaching rate of Cd, Zn, Ni, Cu, Co, Mn, Pb and Cr under optimum conditions 61 to 63 %).

For optimizing the bioleaching process the heavy metal solubilization was tested in laboratory percolators by variation of the sulphur addition to the sediment and of the dosage of sulphuric acid. In the case of sediments free from S^0 only a small portion of heavy metals were solubilized (12 % compared to < 2 % without acid). When adding 2 % S^0, the solubilization rate increased to > 60 %, and the rate was nearly independent of the acidification of the percolating process water.

For a further increase in leaching efficiency, the solubilized metals should be continously removed from the process water without neutralization of the accumulating sulphuric acid. Currently the selective metal removal is being investigated by means of electrochemical methods, membrane filtration and by chemical pecipitation.

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MICROORGANISMS EFFECTS ON SELENIUM VALENCIES

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The most offending long lived fission fragments produced by a standard electronuclear reactor are Tc^{99}, I^{129}, Zr^{93}, Cs^{135}, Sn^{126}, Se^{79}. Earth crust contains a mean selenium content value around 0.09 ppm, with 0.09 to 2 ppm in carbonates, about 0.6 ppm in schists, 0.05 to 1 ppm in sandstones and waters have very variable concentrations. In case of ^{79}Se dissemination, the fate of this radionucleide will be strongly correlated with its carrier the natural selenium and valencies dependent of several parameters (pH, oxygen, water, organic matter, microorganisms). There is a selenium cycle between soil, plants, animals, humans and atmosphere.

Selenium is an essential trace element, and its deficiency is related to several diseases in animals and humans. One explanation for the essentially of the element was found at a molecular level when glutathione peroxydase was identified as a selenoprotein. This enzyme catalyses the reduction of peroxides and thus protects the cells from oxidative damage. However evidence has now accumulated that shows that this is not the only function of the element and that there are several other biologically active selenoproteins. Aerobic microorganisms, as animals and humans, need also a selenium enzyme protection against oxygen free radicals.

Moreover, microorganisms can enzymaticaly reduce a variation of metals in metabolic processes that are not related to metal assimilation. Some can conserve energy to support growth by coupling the oxidation of H_2 or organic material to the reduction of metals. Selenate, selenite may serve as a terminal electron acceptor to support anaerobic growth of some microorganisms so called dissimilatory reduction. Reduction of Selenium (from valence VI to zero) is an important mechanism for the precipitation of selenium from waters and several microorganisms can be involved. Ecological interactions and the potential of such processes for bioremediation of selenium waste waters is a wide spread phenomenon. Furthermore, microbial selenium volatilization (as seleniures and methyl compounds) by microorganisms is an important source for selenium mobilization from soils, sediments, and waters to the atmosphere.

CLASSIFICATION OF *DESULFOVIBRIO* ISOLATES RECOVERED FROM A URANIUM MINING WASTE PILE AND THEIR COMPARISON TO SEVERAL OTHER *DESULFOVIBRIO* STRAINS

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A large variety of bacteria was demonstrated to be present in soil and sediment samples of a uranium waste pile near Johanngeorgenstadt in Saxony, Germany. Anaerobic sulfate-reducing bacteria belonging to the genus *Desulfovibrio* were found among them. These isolates, indigenious for the waste *Desulfovibrio,* were classified by the use of Amplified Ribosomal DNA Restriction Endonucleases (ARDREA), Random Polymorphic Amplified DNA (RAPD), and Repetitive Primer Amplified Polymorphic DNA (rep-APD) analyses. 25 reference strains and five natural isolates were involved in these analyses. Two of them, JG-1 (UFZ B490) and Sediment 5, are presenting a large number of pile isolates recovered from the uranium waste pile. In addition, three other *Desulfovibrio* isolates UFZ B378, UFZ B406, recovered from waste water ponds, and UFZ B393, from a copper mine [3], were analyzed by RAPD and rep-APD.

The reference strains were obtained from the Deutsche Stammsammlung (DSM) and the American Type Culture Collection (ATCC). The total bacterial DNA were isolated by NucleoSpin C+T Kit (Macherey-Nagel).
The ARDREA was performed as described by Selenska-Pobell et al. [7]. The primers 16S-7f and 16S-1541r for the 16S region and 23S-01 and 23S-07 for the IGS region amplification were used. The PCR products were digested with the following restriction enzymes: *Alu*I, *Ava*II, *BstU*I, *Cfo*I, *Dde*I, *Hae*III, *Hin*fI, *Msp*I, *Nde*II, *Rsa*I, and *Taq*I. The resulting patterns were analyzed by the software, RFLP-Scan/Treecon.
RAPD and rep-APD were performed as described by Selenska-Pobell et al. [6]. For the RAPD analysis the primers AP 1 [8], AP 21, and AP 22 [5] were used. The rep-APD analysis was performed using single repetitive primers corresponding to: the A subunit of the BOX element (BOX) [4], to the enterobacterial repetitive intergeneric consensus (ERIC), and to the REP consensus (REP) [1]. The resulting patterns were also analyzed by the RFPL-Scan/Treecon.

The 16S rDNA-RFLP patterns of the pile isolate Sediment 5 were identical to those of the reference strain *D. vulgaris (oxamicus)* 1925^T. However, it was possible to discriminate these strains by ARDREA of the more variable intergenic spacer (IGS) region between the 16S and 23S rDNA genes (Fig. 1 and Fig. 2). Both strains are closely related to the other pile isolate JG-1. The two isolates were recovered from different sites of the same pile.
In addition, three other *Desulfovibrio* isolates UFZ B378, UFZ B406, and UFZ B393 were analyzed in their genomic relationships together with JG-1, Sediment 5, and *D. vulgaris (oxamicus)* 1925^T by RAPD and rep-APD. It was demonstrated that all isolates are members of a group with high genomic similarity. *D. vulgaris (oxamicus)*

1925^T is not very related to these strains. The strains UFZ B378, UFZ B406, and UFZ B393 form a particular group, which differs from the pile isolates JG-1 and Sediment 5. The latter two have almost identical patterns. The close genomic relationships between the five strains studied may be explained by the fact that all of them were cultured from their environmental samples using the same Postgate derived medium with pH 4 as modified by Hard and Babel [2].

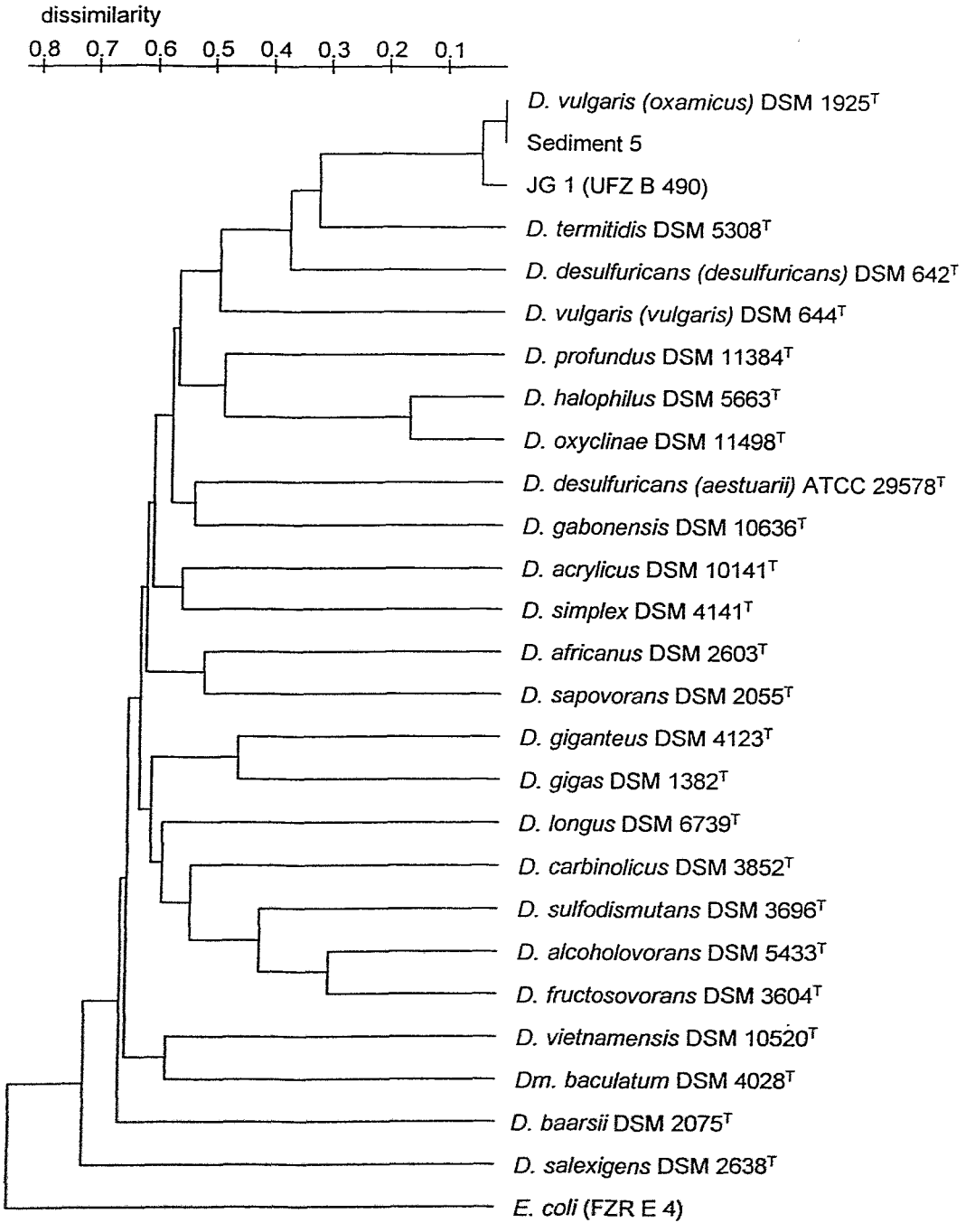

Fig. 1: Dendrogram showing the phylogenetic relationships between the reference strains and the *Desulfovibrio* isolates, JG-1 and Sediment 5, obtained by 16S-ARDREA.

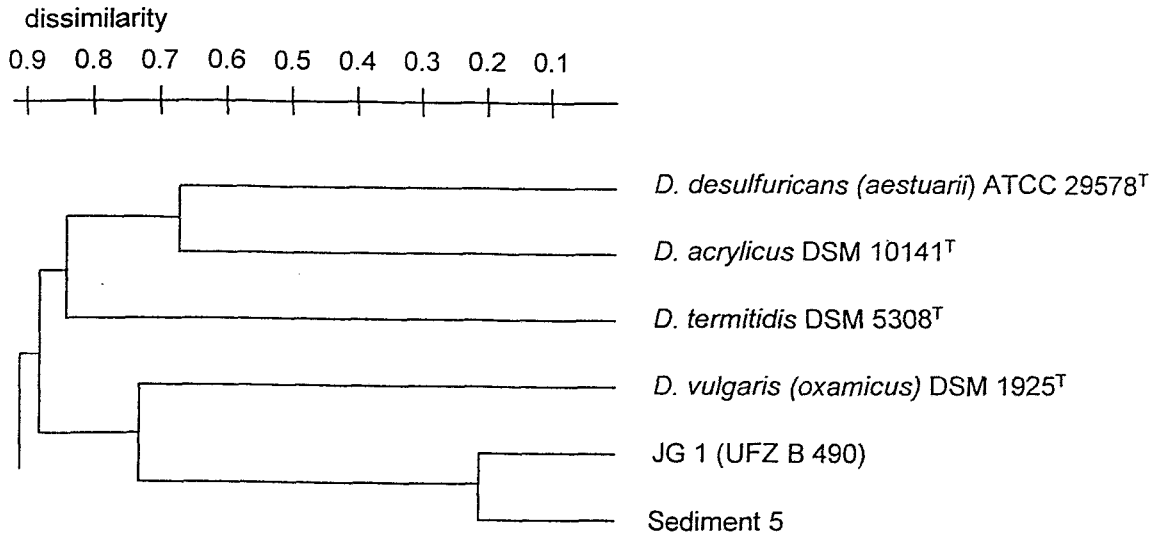

Fig. 2: Dendrogram showing the phylogenetic relationships between the *Desulfovibrio* isolates, JG-1 and Sediment 5, and some reference strains obtained by IGS-ARDREA.

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ENZYMATICALLY-MEDIATED ACTINIDE BIOPRECIPITATION: THE EFFECT OF ANIONIC SPECIES IN THE TARGET SOLUTION

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An atypical *Citrobacter* sp. can accumulate heavy metals and actinide ions via the activity of a cell-bound acid phosphatase that produces HPO_4^{2-} which precipitates with metals as cell-bound metal phosphates. A continuous removal process using immobilized biomass has been applied to the removal of uranium from acidic mine wastewater that contaned also >3000 ppm of SO_4^{2-} [1]. However test columns were run at slow flow rates and for a realistic process to treat large volumes of waste a short flow residence time is necessary. Calculation of the minimum flow residence time, and with this the size of bioreactor required to treat waste on-line, and an economic assessment of process feasibility, require accurate mathematical models of the process. For metal removal from solution this has been achieved (see [1] for references) but the earlier models do not take into account the possibly inhibitory effects of anions present as co-contaminants in the flow. Such ions may act by interfering with the enzymatic process *per se*, or may complex with the target metals, effectively reducing their free concentration in solution and thus increasing the amount of precipitant ligand (here phosphate) required to exceed the metal phosphate solubility product at the cell surface. This can be overcome by generating additional biogenic phosphate to achieve efficient metal precipitation, but the dischage of excess phosphate into the environment is highly undesirable and the secondary waste would itself require treatment. A secondary waste treatment would add to the overal process cost and complexity. A longer flow residence time may be the only approach compatible with the highest process efficiency, and the purpose of this study was to develop and test a mathematical model to determine how the residence time might be affected in the presence of anionic ligands typically-found in actinide-bearing wastes *viz*: SO_4^{2-}, a ubiquitous contaminant of uranium mining wastes, and NO_3^-, derived from the nitric acid used to extract uranium for nuclear fuel fabrication, and in nuclear fuel reprocessing, where the wastes contain not only uranyl ion but also trace amounts of transuranic elements such as Am, Np and Pu. In this study U(VI) was used directly as the model hexavalent actinide species (as UO_2^{2+}) but the toxicity of the artificial transuranic elements precluded their use in the quantites required for process modelling studies. A convenient 'surrogate' for the trivalent transuranic elements (Am and reduced, trivalent, forms of other actinides) is the La^{3+} ion. The tetravalent species were not tested, since they are poorly removed via phosphate precipitation in the presence of complexing ions and their effective removal could be best achieved via pre-oxidation or reduction to more amenable species. Such reduction, achieved

phosphate bioprecipitation route (L.E. Macaskie, P.Yong and J.R. Lloyd, unpublished).

Metal removal by immobilized cells in a flow-through reactor can be accurately described by an adaptation of Michaelis-Menten kinetics which normally describes enzymatic activity in terms of product release (here phosphate) and which can also be used to describe metal removal, since metal and phosphate are precipitated stoichiometrically from solution onto the biomass. By integration of the Michaelis-Menten equation a relationship is obtained which interrelates the flow rate (F: ml/min), the input concentration of substrate (S_o, the source of phosphate, cleaved via enzymatic activity, in mM) and the bioreactor efficiency (X):

$$E_o K_3 = F[S_o X + K_m.\ln(1/(1-X)].$$

X can be expressed in terms of product (phosphate) released or in terms of metal removed. K_m is the Michaelis constant, that substrate concentration (in mM) giving the half-maximal rate of product release, and K_3 is an intrinsic kinetic constant of the enzyme, which can be calculated as a dimensionless term as required. In an immobilized cell reactor, since the enzyme is itself held within the biomass matrix and the biocatalyst may also be subject to diffusional and mass transfer constraints the constants are more correctly described in apparent terms, as $K_{m\ app}$ and $K_{3\ app}$, respectively.

In practice the efficiency of a flow-through reactor operating at steady-state can be expressed as the $FA_{1/2}$ value, which is that flow rate giving 50% of removal of metal from the flow. The effect of an added inhibitor (in this case NO_3^- or SO_4^{2-}) is to decrease the flow rate (i.e. increase the residence time) required to maintain metal removal at the 50% level. The working model would permit the prediction of the residence time 'penalty' for a given concentration of inhibitor under a given set of conditions for removal of a particular 'target' metal species. Since each industrial flow is likely to be different, this generic technology would permit simple benchscale tests to predict the likely cost of the final process.

Initial studies using batch phosphate release tests in the presence of anion showed that the ions were behaving as classical 'competitive' inhibitors of phosphatase activity: the $K_{m\ app}$ was increased (i.e. more substrate was required to maintain a given level of activity). This can be described in terms of the inhibitor constant K_i (specific to each inhibitor) which is effectively the concentration of inhibitor required to decrease the maximum reaction rate (V_{max}) by 50%.

Using this approach with a test bioreactor system using La^{3+} (1 mM) and an excess of nitrate (100 mM; the $K_{i\ app}$ for NO_3^- is 15.9 mM) the model was tested, with experimental data in agreement with that of theoretical predictions, within 10% [2]. Further extension of this approach to the removal of UO_2^{2+} in the presence of excess SO_4^{2-} ($K_{i\ app}$ of SO_4^{2-} is 19.2 mM) shows that the model is generically applicable to bioremediation of actinide/anionic matrices and, as such, is likely to be an invaluable tool in the development and application of bioprocesses to real wastes. In this context the biological removal of Np was demonstrated from nitrate solution at

concentrations of Np and NO_3^- similar to those of some real wastes (J.R. Lloyd, P. Yong and L.E. Macaskie, unpublished).

The question then remains as to remediation of the metal-depleted waste solution. Here, the activity of sulfate-reducing bacteria is well-established in the remediation of SO_4^{2-} laden wastes, while bacterial denitrification is also an established technology, e.g. a novel organism is capable of removing high concentrations of NO_3^- (C. Sahut, pers comm.). The co-application of the nitrate-reducing strain would reduce the level of nitrate concomitantly with metal removal. This would be seen as an increasing rate of *Citrobacter*-mediated metal removal throughout the length of the bioreactor, such that the overall flow rate could be increased, leading to a smaller bioreactor and thus process intensification for more a efficient and integrated process.

References

[1] Macaskie, L.E., Yong, P. Doyle, T.C., Roig, M.G., Diaz, M and Manzano, T. (1997). Bioremediation of uranium-bearing wastewater: biochemical and chemical factors influencing bioprocess application. *Biotechnol. Bioeng.* **53**, 100-109.

[2] Yong, P. and Macaskie, L.E. (1997) Effect of substrate concentration and nitrate inhibition on product release and heavy metal removal by a *Citrobacter* sp. *Biotechnol. Bioeng.* **55**, 821-830.

Last minute information

- Posters **P13, P14,** and **P17** which were initially announced were withdrawn.
- As **P17** a poster will be presented by Christoph Puers and Sonja Selenska-Pobell.
- No abstract for the **P16** from the programme in the final announcement was obtained.