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Pu(IV) and Fe(III) accumulation ability of heavy metal-tolerant soil fungi

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Abstract. The work was aimed at studying abilities of soil microorganisms to participate in metal/radionuclide mobility processes by accumulating them. Soil microorganisms were treated with a mixture of heavy metals (Cr(III), Ni, Fe(III), Mn(II), Cd) in order to isolate the most tolerant ones. Among more metal-tolerant microorganisms microscopic fungi dominated. Tests of fungal tolerance towards each metal showed that the most tolerant fungi to almost all metals were *Aspergillus niger*, *Penicillium oxalicum* and *Paecilomyces lilacinus*. Accumulation ability of metal-tolerant fungi was tested using Pu(IV) and Fe(III). Investigation of Pu accumulation by fungal biomass showed that all the fungi accumulated Pu, and among the most effective radionuclide accumulators *Eupenicillium sp.*, *Penicillium oxalicum* and *Aspergillus niger* could be mentioned. All the fungi showed high Fe-accumulation capacity. While growing in the medium with 1 mM iron, most fungi accumulated over 90% of Fe in their biomass. Very good accumulation and growth abilities in Fe-supplemented medium were demonstrated by *Paecilomyces lilacinus*.

Key words: fungi • Pu(IV) electrodeposition • heavy metals • metal-tolerance

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Introduction

It is well known that microorganisms show a multiplicity of interactions with metals in soil and can contribute to metal mobility or immobilization. The balance between mobilization and immobilization depends on the microorganisms involved, their environment and associated physicochemical conditions [27, 29]. The microbial reduction/oxidation of metals play an important role in the cycling of both inorganic and organic species. Reduction of higher-valency species of metals can lead to mobilization, e.g. Mn(IV) to Mn(II), or immobilization, e.g. Cr(VI) to Cr(III) [16]. Microorganism immobilization of heavy metals can result from sorption, transport and intracellular sequestration or precipitation as organic and inorganic compounds [13, 41]. Interaction of microorganisms with radionuclides also affects radionuclide geochemical processes, in particular radionuclide migration [11, 14, 31]. Ability of microorganisms to immobilize heavy metals and radionuclides has drawn attention of investigators. Investigations are performed searching for microorganisms as effective metal- and radionuclide-accumulators and for their application in removal of heavy metals from polluted substrata [30, 39].

Our study is focused on the investigation of microorganism capabilities of participating in metal/radionuclide mobility processes with special emphasis on their

involvement in redox processes. The aim of the current work was to find out ability of soil microorganisms to survive in metal-polluted environment and influence mobility of metals including radionuclides by accumulating them. The model experiments involved screening of metal-tolerant soil microorganisms, testing their tolerance level and investigation of their metal- and radionuclide-accumulation abilities as the first step in further investigation of redox phenomena.

Materials and methods

Isolation of microorganisms

Grassland soil (400 mg) was treated with a mixture of heavy metals: $MnCl_2 \cdot 4H_2O$ – 22 g, $Cr(NO_3)_3 \cdot 5H_2O$ – 2.2 g, $NiCl_2 \cdot 6H_2O$ – 1.4 g, $Cd(NO_3)_2 \cdot H_2O$ – 0.02 g, $FeCl_3 \cdot 6H_2O$ – 30 g. Metal salts were added as a sterile water solution. The soil was treated for 6 weeks. The control sample was without metal addition, but treated with sterile water of the same amount as the previous one. Microorganisms were isolated on the nutrition agar media (malt extract agar for fungi and Nutrient agar (Liofilchem) for bacteria).

Identification of fungi

Fungi were isolated into pure cultures. For culture isolation and identification, media malt extrct agar, Czapek, Czapek yeast extract agar, potato dextrose agar, and yeast extract sucrose agar were used. Fungi were identified following the handbooks [4, 12, 15, 21, 23, 25, 33, 36–38].

Tests of metal-tolerance of fungi

Fungi were grown on the Czapek yeast agar supplemented with each of the heavy metals (manganese, chromium, nickel, cadmium and iron) as the salts mentioned above, which were used in the range of concentrations from 0.1 to 20 mM. As a control, fungi were grown on the same agar without metals. The plates were three-point inoculated and tests were done in triplicates. Fungi were incubated for 7 days at 26±2°C. The growth of fungi was determined by measuring the diameter of the colonies. The metal effect on fungal growth was evaluated as relative fungal colony growth and expressed in %, which was calculated as d_2/d_1 100%, where d_1 is the mean diameter of the control fungal colonies and d_2 is the mean diameter of the fungal colonies treated with the metals [2, 3].

Investigation of Fe accumulation in fungal biomass

Fungi were grown in a liquid Czapek yeast extract medium. 1 ml of spore suspension (10^6/ml) was added to 50 ml growth medium with 1 mM Fe as $FeCl_3$. Cultures were grown on a rotary shaker at 26±2°C. After 3 day cultivation, the fungal biomass was harvested, dried at 105°C and weighed. Fe remaining in the growth solution was detected by the atomic absorption spectrophotometric (AAS) method using a PERKIN ELMER Zeeman 3030 spectrophotometer. Quality assurance samples (QAS) were obtained from intercalibration exercise which preceded the project "Atmospheric heavy metal deposition in Northern Europe 1990" (intercalibration samples were moss samples prepared for intercalibration purposes). Every 30 samples included two QAS. Acceptable limits were established after examination of intercalibration results [6]. Later on, the reliability of the analysis was proved by appropriate investigations and yearly international intercalibration exercises [35]. For control of the analytical procedure of determination of iron, the intercalibration in the frame of the EMEP project was carried out. MERCK standard solutions were used and the method of calibration was the standard. Results showed an average standard deviation of about 5%.

Evaluation of Pu sorbed by fungal biomass

Fungal cultures were grown in the liquid Czapek yeast extract medium on a rotary shaker at 26±2°C for 3 days. To determine sorbtion ability of plutonium by fungi, 3 day old fungal cultures were treated with 0.0206 Bq of ^{242}Pu(IV) in the medium (100 ml) for 1 h. After the treatment, fungal biomass was dried to constant weight and the dry biomass of pre-cultured microorganisms varied from 0.0621 g to 0.1085 g. Plutonium was extracted from the biomass burnt in a muffle furnace at 550°C overnight by digestion with 8 mol·L^{-1} HNO_3. Plutonium purification was carried out using a strong basic anion exchange resin BiO RaD AG 1 × 8. The electrodeposition method with some modifications is based on the method described by Talvite [40]. Plutonium eluate stripped from the anion exchange column is evaporated and added 1ml of Na_2SO_4 (0.3 mol/l) is evaporated as well. Then 300 µl of conc. H_2SO_4 is added and heating is performed until the sample is dissolved. After that, 4 ml of H_2O and 5 ml of 1% H_2SO_4 are added. The pH value of 2.1–2.4 is achieved with ammonia (if the end point is exceeded, 20% H_2SO_4 is used for correction). Thus, the volume of the electrolyte amounts to about 10 ml. The cathode planchets were made from the stainless steel 7C27Mo2 Sandvic [1]. The exposed cathode (polished stainless steel planchet) area was 2.3 cm^2. The anode used for electrolysis was a platinum wire of horizontal helix shape that is adjusted to the cathode planchet to about 3 mm. Electrolysis at 1A lasts for 1 h and electrodeposition yield comes to 98%. Each series of the study begins from the blanks. The chemical yield attained by the use of this analytical procedure for ^{239,240}Pu determination in the environmental samples (^{242}Pu applied as an internal tracer standard) was in the range of 60–80%. Plutonium isotopes are determined by alpha-spectrometry using a Canberra PD type detector (area 450 mm^2, resolution 17 keV (FWHM) at 4–6 MeV). The detection limit for the counting time of 86,400 s is about 10^{-3} Bq for ^{239,240}Pu.

The long-lasting investigations of ^{239,240}Pu spatial distribution in the top soil layer [26] and in the lake bottom sediments [34] using the above-mentioned method have been carried out. Performance of the method was

tested within the worldwide Intercomparison Exercise IAEA-385 "Radionuclides in Irish Sea sediment" and a good agreement of the results was obtained [32].

Results and discussion

The experiment of heavy metal effect on microorganisms showed that after 6 weeks the mixture of heavy metals evidently influenced microbial counts and their community structures. Especially severe effect of the metals was exerted against bacteria. Only ~0.1% of the total count of bacteria remained compared with the control. Fungi showed significantly higher tolerance to heavy metals. About 61% of the total count of fungi survived in the metal-treated soil. Reduction in microbial counts indicates that essential functions of microorganisms have been affected or cells have been damaged [8, 20]. Evidently, the species composition of fungi in soil affected by metals was poorer than the control sample. Fungi such as *Aspergillus niger*, *Fusarium sp.*, *Eupenicillium sp.*, *Penicillium oxalicum*, *Paecilomyces lilacinus* and *Phoma sp.* were among the best survived fungi, and were further investigated for their metal-tolerance and accumulation abilities. These fungi are common in soil and could be expected to be distributed at repository sites. Additionally, it is reported that fungi of these taxonomical groups such as *Aspergillus niger*, *Penicillium*, *Paecilomyces*, *Fusarium*, were spread in locations with radioactive contamination [43].

Some other studies also showed a change in the microbial community structure in the metal polluted environment indicating a bacterial population decrease and shift towards fungi [7, 22, 24]. Rather high metal-tolerance of fungi, determining their survival in polluted substrata, can be related to the fungal intrinsic peculiarities including the cell wall composition, extracellular polysaccharide and metabolite excretion that lead to binding or precipitation of metals [5, 9].

Tests of metal-tolerance showed that these fungi differed in their tolerance reaction towards each metal of the mixture. The most negative influence on the fungal growth was exerted by cadmium and nickel, while manganese was the least harmful to most of cultures (Fig. 1). Growing in the medium with Cd, all fungi were able to develop at 1 mM Cd, and 2.5 mM of the metal

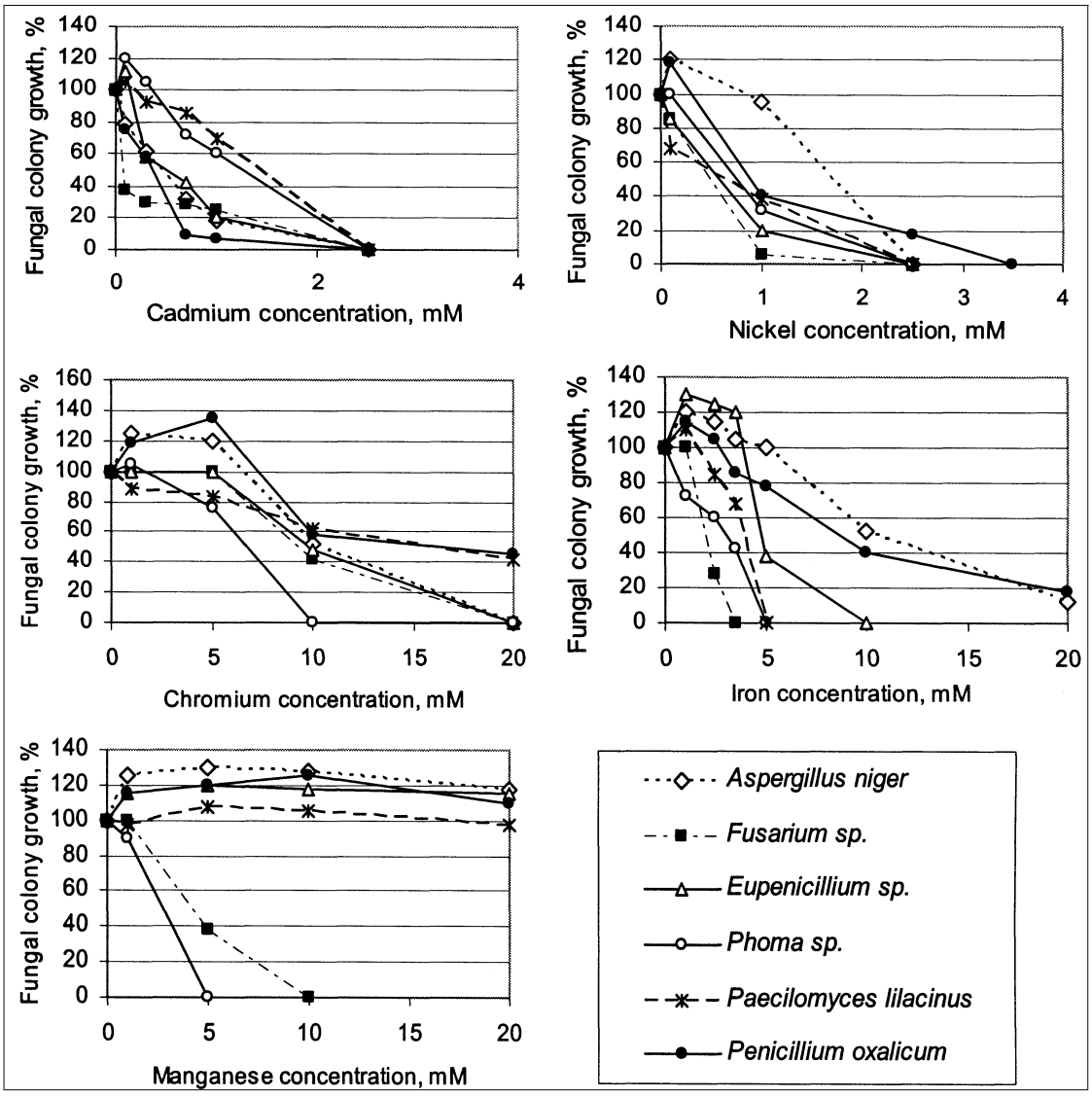

Fig. 1. Growth of fungi on the metal-containing media, represented as fungal colony diameter after 7 day growth compared to fungal colonies on metal free-media.

Table 1. Fe removal from growth medium by growing fungal biomass (Fe added to the medium – 1 mM)

Fungi	pH change (decrease), $pH_{initial}$ = 7.15	Accumulated Fe, mg/g dry biomass	Fe removed from the growth medium (%)	Biomass growth (compared to the control growth without iron addition) (%)
Aspergillus niger	–3.48	16.7	82.8	107
Eupenicillium sp.	–2.74	30.7	93.0	104
Fusarium sp.	–1.31	51.0	96.0	77
Paecilomyces lilacinus	–0.92	38.7	98.7	113
Phoma sp.	–0.84	29.4	99.7	76
Penicillium oxalicum	–0.92	20.6	94.8	98

was lethal to the fungi. Tolerance to nickel was rather similar, however *Penicillium oxalicum* was able to grow at 2.5 mM of Ni. The most tolerant fungi to Cr were *Penicillium oxalicum* and *Paecilomyces lilacinus*, which grew at 20 mM in the medium. Chromium exhibited the most negative effect on *Phoma sp.* – 10 mM Cr absolutely inhibited growth of the fungus. Evident differences in tolerance were noticed when Fe was added to the medium. The most sensitive fungus was *Fusarium sp*., which was unable to develop at 3.5 mM Fe, whereas *Penicillium oxalicum* and *Aspergillus niger* developed even at 20 mM of Fe in the medium. Manganese showed the weakest negative effect on the fungi. Most of the microorganisms grew very well even at 20 mM manganese in the medium, only two of them *Phoma sp*. and *Fusarium sp.* were much more susceptible to the metal. High tolerance of *Penicillium*, *Aspergillus* and *Paecilomyces genera* was also shown by other studies [19, 42].

The metal-tolerant fungi were investigated for heavy metal (Fe) and radionuclide (^{242}Pu) accumulation abilities. Iron and plutonium have chemical and biochemical similarities, and microorganisms participating in Fe mobility regulation could be important in Pu solubility and bioavailability [17]. The results showed that while growing in the medium the fungi were able to accumulate significantly high amounts of Fe by their biomass (Table 1). Fungi *Phoma sp.* and *Paecilomyces lilacinu*s removed the highest Fe amount from the media – 99.7 and 98.7%, respectively. However, the biomass amount of these two fungi differed greatly. The growth of *P. lilacinus* was even stimulated by added Fe (compared to the control growth in Fe-free medium), while the growth of *Phoma sp.* was affected negatively. When calculating Fe amount accumulated in the biomass, the highest accumulation capacity was demonstrated by *Fusarium sp*. (51 mg/g dry biomass). However, the growth of this fungus was suppressed by Fe.

A rather high Fe accumulation capacity was shown by *P. lilacinu*s and *Eupenicillium sp*. It should be mentioned that 1 mM Fe had no negative effect on the biomass growth; on the contrary, slight growth stimulation was noticed. The least Fe amount removed from the medium was detected in *Aspergillus niger* case. The fungus grew very well in a Fe-supplemented medium, whereas its accumulation capacity was much weaker as compared to the other fungi. The pH of the growth medium was shifted to the acidic side by all the fungi and most evidently by *Aspergillus niger*, known as a producer of acidic metabolites [28]. Thus, summarizing the most efficient Fe accumulators, which also tolerated the metal added well, were *Paecilomyces lilacinu*s and *Eupenicillium sp.*

Table 2. Plutonium(IV) sorbed by fungal biomass during 1 h from the liquid medium supplemented with ^{242}Pu

Fungi	^{242}Pu, Bq/g dry biomass
Aspergillus niger	0.0335
Eupenicillium sp.	0.0514
Fusarium sp.	0.0165
Paecilomyces lilacinus	0.0093
Phoma sp.	0.0099
Penicillium oxalicum	0.0249

The investigation of ^{242}Pu accumulation by metal-tolerant fungi showed that all the fungi were able to sorb the radionuclide ^{242}Pu by their living biomass (Table 2). The least radionuclide amount sorbed during 1 h treatment was 0.0093 and 0.0099 Bq/g of dry biomass of *Paecilomyces lilacinus* and *Phoma sp*., respectively. The highest radionuclide sorption capacity was detected in *Eupenicillium sp.* biomass (0.0514 Bq/g). Fungi *Aspergillus niger* and *Penicillium oxalicum* also showed good sorption abilities – 0.0249 and 0.0325 Bq/g of ^{242}Pu were detected in their biomass.

Microorganisms could adsorb radionuclides on their cell surfaces or accumulate them within cell. A high plutonium sorption capacity by biomass was also demonstrated using fungus *Rhizopus arhizus*, which removed significant amounts of plutonium [10]. Sorption as an immobilization process may make unable metals and radionuclides to be transformed *in situ* into insoluble forms and could be applicable to remove them from polluted aqueous solution [18].

Conclusions

The obtained results show that soil fungi are able to sorb ^{242}Pu and Fe rather effectively. Additionally, high heavy metal tolerance of soil fungi should be emphasized, especially when compared to bacteria. This peculiarity of fungi could help to survive them in higly polluted habitats. The most tolerant microorganisms towards heavy metals added to the soil in our study were *Aspergillus niger, Fusarium sp., Eupenicillium sp., Penicillium oxalicum, Paecilomyces lilacinus* and *Phoma sp.*

Metal-accumulation experiments revealed that metal-tolerant fungi were able to remove Fe from the solution highly efficiently – most fungi accumulated over 90% of Fe in their biomass. Very good accumulation and growth properties in Fe-supplemented medium were demonstrated by *Paecilomyces lilacinus* and *Eupenicillium sp*. Investigation of ^{242}Pu accumulation by fungal biomass showed that all the metal-tolerant fungi

were able to sorb ^{242}Pu in their biomass from 0.0093 to 0.0514 Bq/g of dry biomass. Fungi *Eupenicillium sp*., *Penicillium oxalicum* and *Aspergillus niger* showed the most effective radionuclide ^{242}Pu sorption.

Abilities to accumulate high amounts of the metals and radionuclides allow fungi to be active participants in metal migration processes in polluted habitats, including repositories. Microbial biosorption capacities of heavy metals and radionuclides could also have potential application in bioremediation of the polluted environment.

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