Microbial removal of toxic metals from a heavily polluted soil

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A B S T R A C T

Samples of a leached cinnamonic forest soil heavily polluted with uranium and some toxic heavy metals (mainly copper, zinc and cadmium) were subjected to cleaning by means of bioleaching with acidophilic chemolithotrophic bacteria. The leaching of the soil was performed by stimulating the activity of these bacteria to leach and remove the pollutants from the horizon A to the deeply located horizon B_2 in which pollutants were precipitated by stimulating the activity of the indigenous sulphate-reducing bacteria. The treatment was carried out in a green house in which several plots containing 150 kg of soil each were constructed. The effect of some essential environmental factors such as pH, humidity, temperature and contents of nutrients on the cleaning process was studied. It was found that under optimal conditions the content of pollutants were decreased below the relevant permissible levels within a period of 170 days. The soil cleaned in this way was characterized by a much higher production of biomass of different plants (alfalfa, clover, red fescue, vetch) than the untreated polluted soil.
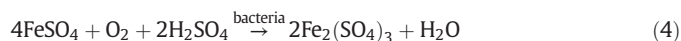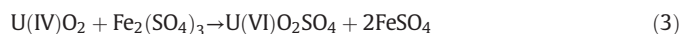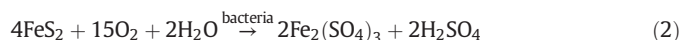
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1. Introduction

The pollution of waters and soils by toxic heavy metals is a serious environmental problem in many countries, especially in these with intensive industrial development and/or with a large-scale recovery of such metals from the relevant mineral deposits. The pollution is due to different mechanisms, some of which are acting under natural conditions but others are directly connected with the human activity. In some cases this activity is connected with the recovery of heavy metals from the relevant natural sources, mainly the ore deposits, but in some cases the recovery is connected with the processing of some mineral wastes or even of industrial products such as metal-bearing concentrates. Since a relatively long period of time some of these technologies are connected with the application of different microorganisms able to solubilize or to precipitate the relevant metals under suitable conditions. Apart from the recovery of different metals, some microorganisms are used to prevent the pollution or even to participate in the cleaning of the ecosystems polluted by toxic metals.

In Bulgaria, for a long period of time uranium was leached commercially in a large number of deposits using mainly different in situ technologies. Most of these commercial-scale operations were connected with the acid leaching of uranium due to the presence of pyrite and the negative net neutralization potential of the relevant uranium ores. In some of these operations the leaching was connected with the action of some acidophilic chemolithotrophic bacteria which were able to oxidize the tetravalent uranium to the soluble hexavalent form and to generate sulphuric acid and ferric ions by the oxidation of pyrite present in such deposits (reactions 1–4):

$$U(IV)O_2 + 0.5O_2 + H_2SO_4 \overset{bacteria}{\rightarrow} U(VI)O_2SO_4 + H_2O \quad (1)$$

$$4FeS_2 + 15O_2 + 2H_2O \overset{bacteria}{\rightarrow} 2Fe_2(SO_4)_3 + 2H_2SO_4 \quad (2)$$

$$U(IV)O_2 + Fe_2(SO_4)_3 \rightarrow U(VI)O_2SO_4 + 2FeSO_4 \quad (3)$$

$$4FeSO_4 + O_2 + 2H_2SO_4 \overset{bacteria}{\rightarrow} 2Fe_2(SO_4)_3 + H_2O \quad (4)$$

Several years ago, all commercial-scale operations for uranium leaching in the country were stopped due to a complex of different political, economical and environmental reasons. Regardless of some preventive and remedial actions during the uranium recovery, many natural ecosystems were heavily polluted with radioactive elements and several toxic metals, mainly through the seepage of acid drainage waters. Soils around the water flowpath were polluted with these toxic elements and some of them are still unsuitable for agricultural use.

It is known that different methods for assessment and remediation of soils contaminated with uranium and toxic heavy metals are available (Komnitsas and Modis, 2006; Groudev et al., 2001, 2008, 2010; Park et al., 2011; Malavija and Singh, 2012; clean et al., 2013; Singh et al., 2014; Asselin and Ingram, 2014; Ma et al., 2015; Romero-Freire et al., 2016). However, only few of them have been applied under real large-scale conditions. The excavation and transportation of the heavily polluted soils to specific depositories is still a common practice in most

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countries. In some cases the disposal is followed by off-site treatment of the relevant soils. The in situ monitored natural attenuation or passive capping using the installation of clean, inert material over the contaminated soil are also largely applied. The application of methods for remediation in situ of soils contained with toxic heavy elements (such as heavy metals, uranium and arsenic) is still limited but can be very attractive especially from economical point of view. The in situ leaching is connected with solubilization of uranium and heavy metals by means of different chemical lixiviants (bicarbonate, mineral acids and some organic complexing agents) or by means of different microorganisms, mainly acidophilic chemolithotrophic bacteria able to oxidize the insoluble tetravalent uranium to the soluble hexavalent form. The bacterial leaching is especially efficient in the cases when the metals are present in the form of the relevant sulphide minerals and in some cases the soil remediation can be connected with the recovery of the dissolved metals from the relevant pregnant leach solutions.

Another group of in situ bioremediation methods are connected with the immobilization of uranium and heavy metals inside the soil by converting them into their least soluble or toxic forms or by encapsulation in solid products of high structural integrity (Mulligan et al., 2001). The anaerobic sulphate-reducing bacteria are especially effective in this respect since in the presence of suitable electron donors, mainly different biodegradable organic compounds but also hydrogen, they are able by the process of dissimilatory sulphate reduction to precipitate the dissolved heavy metals as the relevant insoluble sulphides, and the dissolved hexavalent uranium as the insoluble tetravalent form.

The efficient remediation of contaminated soils under real scale and climatic conditions is connected with the detailed controlled conditions. Data about such testing of biotechnology for remediation of heavily contaminated soils are present in this paper.

The source of the soil used in this study was a plot of land located in the proximity of the Curilo village, Bulgaria. This plot since a long period of time was used for agriculture and as a pasture for domestic animals. The activities connected with the mining and recovery of uranium starting after the Second World War in this area caused considerable changes in the character of the nature, including of that of the plot mentioned above. The soil in this plot before the start of the mining activities was a typical cinnamonic forest soil, the changes in the plot were connected mainly with the pollution of the soil, especially of the horizon A, i.e. to a depth of about 30 cm from the surface, by radionuclides (mainly uranium and radium) and toxic heavy metals (mainly copper, zinc and cadmium). These pollutants were transported to the plot mainly by the acid drainage waters generated in the dumps located in the proximity of this plot and consisting of rich-in-pyrite mining wastes. Considerable amounts of the pollutants were transported also under the form of fine particles removed from the dumps and deposited on the soil by means of the air transportation. It must be noted that apart from the deep changes in the chemical composition of the soil as a result of the pollution, the initial biocenose of the non-polluted soil was also considerably changed. The polluted soil was still rich-in-organics but the species composition and the numbers of the typical soil heterotrophic microorganisms, both bacteria and fungi, were considerably decreased, as well as the numbers of the typical representatives of the protozoa and algae. The typical members of the mesobiota in the non-polluted soils of this type, such as nematodes, oligochaete worms, insects larvae and microarthropods, were also considerably decreased. The negative changes covered also different representatives of the local macrobiota, including the roots of plants, the larger insects, earthworms and even some burrowing vertebrates such as moles and different rodents. These changes made the former suitable for agriculture land unsuitable and even dangerous for normal human activities and were the reason for the future remediation of the contaminated soils in this area.

It is known that different methods for remediation of soils contaminated with uranium and toxic heavy metals are available (Groudev et al., 2008, 2010). In the past the treatment of such soils

was connected with their removal by extraction followed by off-site treatment/disposal, as well as with the in situ monitored natural attenuation or passive capping using the installation of clean, inert material over the contaminated soil.

In situ management of contaminated soils is potentially less expensive and less risky than ex situ management (Knox et al., 2008). In situ leaching is connected with the solubilization of the uranium and heavy metals by means of different chemical lixiviants (bicarbonate, mineral acids and some organic complexing agents) or by means of different microorganisms, mainly acidophilic chemolithotrophic bacteria, able to oxidize the insoluble tetravalent uranium to the soluble hexavalent form. The bacterial leaching is especially efficient in the cases when the metals are present in the form of the relevant sulphide minerals and in some cases the soil remediation can be connected with the recovery of the dissolved metals from the relevant pregnant leach solutions.

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2. Materials and methods

The treatment of contaminated soil was carried out by using plots containing 150 kg of soil (dry weight) each. The plots had a quadratic form with sides of 0.6 m. The soil profile consisted of horizon A 30 cm, horizon B_1 20 cm, horizon B_2 30 cm, and horizon C 20 cm (the real horizon C in the land plot near the Curilo deposit was about 80 cm in depth). The soil initially contained its own microflora and mesobiota, and the temperature and water regimes during the treatment were similar to these of the soil in the real land plot.

The soil treatment in plots № 1 and № 2 was connected with the initial solubilization of contaminants present in the top layers, mainly in the horizon A. The natural microflora of theses plots contained some acidophilic chemolithotrophic bacteria, mainly of the species *Acidithiobacillus ferrooxidans, Acidithiobacillus*

Table 1
Characteristics of the soil horizon A in the plots before and after the treatment.

Parameters	Before treatment	After treatment
Chemical composition, %		
SiO_2	77.6	78.7
Al_2O_3	12.5	11.8
Fe_2O_3	2.48	1.54
P_2O_5	0.15	0.12
K_2O	2.21	1.60
N total	0.10	0.09
S total	1.81	0.77
S sulphidic	1.56	0.65
Carbonates	0.16	0.02
Humus	2.15	1.52
pH (H_2O)	4.39	3.12
Net neutralization potential, kg $CaCO_3$/t	−45.0	−20.8
Bulk density, g/cm³	1.34	1.27
Specific density, g/cm³	2.70	2.59
Porosity, %	53	48
Permeability, cm/h	10.9	8.8
Partical size, mm (%)		
1.00–0.25	19.4	18.3
0.25–0.01	50.1	51.5
<0.01	30.5	30.2

Table 2
Microorganisms in the experimental soil plots before and after the treatment.

Microorganisms, cells/g dry soil	Horizon A		Horizon B2	
	Before treatment	After treatment	Before treatment	After treatment
Aerobic heterotrophic bacteria	10^6	10^4	10^3	10^3
Acidophilic chemolithotrophs	10^4	10^7	10^2	10^2
S^o – oxidizing chemolithotrophs at pH 4–9	10^5	10^4	10^3	10^2
Nitrifying bacteria	10^4	10^2	10^2	10^1
Fungi	10^4	10^3	10^3	10^2
Anaerobic heterotrophic bacteria	10^3	10^2	10^4	10^5
Sulphate-reducing bacteria	10^2	10^1	10^3	10^5
Denitrifying bacteria	10^2	10^1	10^3	10^3
Fe^{3+}-reducing bacteria	10^2	10^1	10^2	10^2

thiooxidans and *Leptospirillum ferrooxidans,* with small numbers of some microorganisms (mainly heterotrophic bacteria and fungi) typical for some relatively slightly polluted soils in the same plot.

Water acidified with sulphuric acid to pH in the range of 2.8–3.0 was used as leach solution. Periodically, this solution was supplemented with ammonium and phosphate ions in concentrations sufficient to maintain their concentrations in the soil pore solution in the ranges of about 20–30 and 10–15 mg/L, respectively. The irrigation rate and acidity of the leach solution were adjusted in connection with the relevant levels in the real soil located near the Curilo deposit. The upper soil layers were periodically subjected to digging to enhance the natural aeration. In this way the concentration of oxygen dissolved in the pore solution in the horizon A and even in the upper part of the horizon B_1 was maintained at values higher than 3 mg/L. However, the conditions in the horizon B_2 were anaerobic, with negative Eh potentials, (usually down to minus 50–70 mV) and a relatively higher pH (>3.0). These conditions allowed most of the dissolved contaminants to be removed from the soil profile of these two plots through the soil effluents.

The treatment of the horizon A of the soil plots № 3 and № 4 proceeded under the same conditions as these in the horizon A of the soil plots № 1 and № 2. However, the horizon B and, more especially, the horizons B_2 of these soil plots were maintained as typical anaerobic systems with negative Eh potentials (within the range of about minus 150 to minus 230 mV) and with pH higher than 4.5. This was achieved by injecting water solutions of organic compounds (lactate and acetate) and ammonium and phosphate ions, with pH of about 4.5–5, through vertical boreholes to this soil horizon. In this way, the conditions in the horizons B_2 were suitable for the growth and activity of the sulphate-redusing bacteria.

The soil treatment in the soil plot № 5 was carried out by irrigation the soil with more acidic leach solutions (with pH about 1.8–2.0) and a more intensive aeration to maintain the content of dissolved oxygen at concentrations higher than 5 mg/L. These solutions also contained the dissolved ammonium and phosphate ions as nutrients for the microorganisms inhabiting this soil plot.

The main geotechnical characteristics of the soil plots, such as permeability and wet bulk density, were measured in situ using the sand-core method (U.S. Environmental Protection Agency, 1991). True density measurements were carried out in the laboratory using undisturbed core samples. Such samples were also used for determination of their acid generation and net neutralization potentials using static acid-base accounting tests. The bioavailable fractions of the contaminants were determined by leaching the samples with DTPA and ADTA (Sobek et al., 1978). The mobility of the contaminants was determined by the sequential extraction procedure (Tessier et al., 1979). The toxicity of soil samples was determined by the EPA Toxicity Characteristics Leaching Procedure (U.S. Environmental Protection Agency, 1990).

The isolation, identification and enumeration of microorganisms were carried out by method described elsewhere (Karavaiko et al., 1988; Groudeva and Tzeneva, 2001; Hallberg and Johnson, 2001; Escobar et al., 2008; Sanz and Köchling, 2007).

The ecotoxicity of the soils before, during and after the treatment towards different test-organisms was determined by the methods described elsewhere (Groudev et al., 2007).

3. Results and discussion

The leaching of contaminants in the horizon A of the plots was efficient and within a period of 170 days (including 68 days of soil irrigation and 102 days of pauses between some of the subsequent irrigations) (Table 1). The leaching was connected with an intensive growth and activity of the indigenous acidophilic chemilithothrophic bacteria which number increased from the initial 10^4 cells/g dry soil to >107 cells/g at the end of leaching. Most of these bacteria were attached on the soil particles, including on the sulphide minerals present in the contaminated soil (mainly pyrite but also some copper-bearing sulphides such as chalcopyrite, covellite and chalcocite). *Acidithiobacillus ferrooxidans* was the prevalent microorganism during the leaching. This bacterium oxidized the sulphide minerals, as well as the S^0, the reduced inorganic sulphur compounds, the tetravalent uranium and ferrous iron present in the soil. *Leptospirillum ferrooxidans* and *Acidithiobacillus thiooxidans* were also present but in lower numbers than *At. ferrooxidans*. *L. ferrooxidans* oxidized directly the Fe^{2+} ions to Fe^{3+}, and the sulphide minerals and U^{4+} indirectly by the Fe^{3+} ions. *At. thiooxidans* oxidized S^0 to sulphuric acid very efficiently, usually at higher rates than *At. ferrooxidans*. Mixed cultures of *L. ferrooxidans* and *At. thiooxidans* oxidized sulphide minerals at rates similar to these obtained by *At. ferrooxidans*.

The pH of the soil was decreased from the initial 4.39 to 3.12 during the leaching and the content of carbonates was also decreased due to their solubilization by the sulphuric acid present in the leach solutions and also generated as a result of the bacterial oxidation of pyrite. The bacterial generation of acidity in situ in the horizon A not only decreased the quantity of sulphuric acid added from outside to the leach solutions but, together with the decrease of the soil pH, facilitated also the decrease of the acid-consuming minerals (mainly carbonates) present in this soil horizon. The increase of acidity and decrease of the acid-consuming minerals resulted in the increase of the net neutralization potential of the soil horizon A to values more close to the neutral point. These changes transformed this soil horizon into an ecosystem

Table 3
Contents of contaminants in the horizon A of the soil plots before and after the treatment.

Contaminants	Before treatment	After treatment					Permissible levels for soils with pH	
		Plot No 1	Plot No 2	Plot No 3	Plot No 4	Plot No 5	<4.1	4.1–5.0
Content of contaminants, ppm								
Cu	159	32	37	35	31	19	20	40
Zn	196	41	55	51	44	28	30	60
Cd	4.8	0.4	0.7	0.4	1.2	0.4	0.5	1.5
U	62	9.0	10.4	8.2	9.5	7.1	10	10
Ra	440	50	55	50	60	50	65	65

Table 4
Contents of contaminants in the horizon B of the soil plot No 1 before and after the treatment.

Parameters	Cu	Zn	Cd	U
Contents before treatment, mg/kg	71	88	1.7	23
Contaminant transferred from the horizon A, mg/kg	127	155	3.1	53
Maximum possible cumulative content, mg/kg	76.2	93	1.86	54.8
Real content after treatment, mg/kg	44	53	1.0	14
Decrease of the contaminant content in the horizon B, %	38.0	39.8	41.2	39.1
Removal from the soil profile (horizon A + horizon B), %	62.0	62.3	64,2	67.8

relatively more suitable for acidophilic chemolithotrophic bacteria and stimulated the bacterial oxidation of sulphides and uranium. This oxidation was connected with the solubilization of the relevant toxic heavy metals and facilitated their removal from the horizon A by the drainage leach solutions. The content of humus also decreased as a result of leaching (from the initial 2.15% to about 1.52% in the horizon A at the end of the leaching). Considerable portions of the contaminants were removed from the horizon A during the leaching but their temporary presence in soluble form also increased the toxicity of the relevant soil plot. All these changes resulted in a temporary decrease of the contents of the typical soil microorganisms, mainly heterotrophs, in the soil plots subjected to leaching (Table 2).

Portions of the contaminants solubilized in the horizon A were removed from the soil profile by the relevant effluents (from the plots No 1 and No 2 but especially from the plot No 5) (Table 3). However, considerable portions from the dissolved contaminants were precipitated in the soil horizon B (mainly in its deeply located part, i.e. in the subhorizon B_2) as the relevant insoluble sulphides as a result of the activity of the sulphate-reducing bacteria inhabiting this anaerobic soil subhorizon (mainly in the plots No 3 and No 4) (Table 4). The insoluble hexavalent uranium was reduced to the tetravalent state and precipitated as the relevant insoluble uraninite (UO_2). These processes were a result of the enhanced activity of the indigenous sulphate-reducing bacteria and their number during the treatment was increased from the initial 10^3 cells/g dry soil to about 10^5–10^6 cells/g at the end of the treatment. Some of these bacteria were active even at pH lower than 4.0. This is not surprising since acidophilic sulphate-reducing bacteria are known since a long period of time (Sen and Johnson, 1999) and such bacteria related to the species Desulfosporosinus acidianus were recently isolated from acidic sediments (Sanchez-Andrea et al., 2015). It must be noted that relatively small portions of the dissolved non-ferrous metals and uranium were removed from the drainage waters by means of sorption on the soil particles and by the hydroxides and oxides of iron and manganese present in the soil. However, the sorption was an essential mechanism for the removal of radium from these waters. The initially adsorbed ions of the non-ferrous metals and uranium were then subjected to precipitation as the insoluble sulphides and uraninite, respectively.

Table 5
Toxicity of the soil in the plot No 1 before and after the treatment and after the remediation.

Test-organisms	Toxicity		
	Before treatment (pH 4.39)	After treatment (pH 3.12)	After remediation (pH 5.90)
Bacillus cereus	40	35	90
Pseudomona putida	30	30	NOEC at 100
Lumbricus terrestris	25	15	75
Trifolium pratense	35	30	NOEC at 100
Medicago sativa	35	30	90
Festuca rubra	30	25	NOEC at 100
Lactuca sativa	40	35	NOEC at 100

Notes: The toxicity was expressed as the lowest observed effect concentration (LOEC) at different contents (in wt%) of contaminated soil in a mixture with clean soil of the relevant type.
NOEC – no observed effect concentration.

Table 6
Production of plant biomass from the soil plots before and after the treatment.

Plants	Biomass recovery (dry weight/m²)	
	Before treatment	After treatment
Clover (Trifolium pratense)	370–505	680–752
Alfalfa (Medicago sativa)	`325–480	735–815
Vetch (Vicia sativa)	215–325	675–735
Red fescue (Festuca rubra)	235–350	590–684

It was found that the toxicity of the soil was connected mainly with its pH and the concentrations of contaminants in the soil pore solutions. The toxicity steadily increased during the leaching (Table 5). However, after the end of leaching connected with the decrease of the contents of toxic metals in the soil, and followed by its remediation by adding nutrients (mainly assimilable sources of nitrogen and phosphorous) and limestone (to increase the pH close to the neutral point), the toxicity of the soil was considerable decreased. This resulted in considerable increase of the amount of plant biomass produced after the soil cleaning in these soil plots (Table 6).

4. Conclusions

The system used in this study for remediation of an acidic soil heavily contaminated with radionuclides and heavy metals was based on the monitored stimulation of the growth and activity of the natural microflora of the two soil horizons – A and B_2. The idea was to solubilize and remove the contaminants from the most essential from the agrobiological and ecological points of view soil horizon A, and to transfer and immobilize them in the deeply located soil horizon B_2. This was achieved by enhancing the growth and activity of the acidophilic chemolithotrophic bacteria inhabiting the top soil horizon A by suitable changes of some environmental factors to values more favorable for these bacteria: the acidification of the soil pH to 2.8–3.0 by sulphuric acid and as a result of the bacterial oxidation of sulphides (mainly of the pyrite) present in this soil horizon, the increasing of the horizon aeration for these aerobic bacteria by periodic digging of the soil, and by adding of ammonium and phosphate ions as essential nutrients. The contaminants solubilized in this way, mainly as the relevant sulphates, were transferred by the drainage acidic solutions to the deeply located soil horizon B_2 in which the heavy metals were precipitated as the relevant insoluble sulphides, and the soluble hexavalent uranium was reduced and precipitated as its tetravalent form. The precipitation of contaminants was due to the process of microbial dissimilatory sulphate reduction performed by the anaerobic sulphate-reducing bacteria inhabiting this anoxic soil horizon. The growth and activity of these bacteria were enhanced by adding via boreholes to this horizon via boreholes solutions of biodegradable organic compounds (lactate and acetate) which were used as donors of electrons in the process above mentioned.

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