BIOTECHNOLOGIES FOR REMEDIATION OF SOILS POLLUTED WITH HEAVY METALS BY ACID MINE DRAINAGE

PLAMEN GEORGIEV[*], STOYAN GROUDEV, IRENA SPASOVA, MARINA NICOLOVA

Department of Engineering Geoecology, University of Mining and Geology "St. Ivan Rilski", Sofia Bulgaria
Corresponding author: ps_georgiev@mgu.bg

Keywords: heavy metals, leaching, immobilization, soil

Abstract: Acid mine drainages (AMD) are generated as a result of bacterial oxidation of sulphides in the mining area. Acidic water pH, higher concentrations of iron, non-ferrous metals, radioactive or toxic elements are present, mainly as free ions highly toxic to organisms living in surrounding aquatic and terrestrial ecosystems, are the reason why AMD is regarded as one of the most complex environmental problems arising due to the human activity.

Remediation of AMD-affected soil was studied by means of two completely different biotechnological methods. The pollutants leaching from topsoil as a result of addition of S° and straw to the soil was the aim of the first method. As a result, growth and activity of sulfur-oxidizing bacteria and fungi were stimulated and due to the lowering soil pH and higher concentration of organic acids into the soil solutions, the soil pollutants were easily leached from the topsoil. At the end of soil remediation, the concentration of the soil pollutants was decreased below the relevant Maximum Admissible Concentrations (MAC) for heavy metals in soils. Soil reclamation was applied as a next stage with the aim to restore soil pH and increase the organic content of the soil.

The other method of soil remediation and reclamation was based on the pollutants' immobilization into the topsoil. It was achieved by means of the addition of a mixture with C:P = 150:1 consisting of carbonate, hydroxyapatite ($Ca_{10}(PO_4)_6OH$), compost, and montmorilonite. Due to the phosphate-releasing process and consumption of H^+ by means of chemical and microbial processes, the heavy metals were precipitated as sparingly soluble phosphates and soil pH was increased to 6.18. As a result, the toxicity of AMD-affected soil to organisms was decreased between five to eight folds in comparison with non-treated soil.

INTRODUCTION

Remediation of soils polluted by radioactive elements and non-ferrous metals could be divided into two main groups in respect to the pollutant's behavior during the remediation process. The first group includes methods where the main goal is to stop/ prevent the pollutant migration into the environment. It could be achieved by means of their transformation into solid phases with higher stability due to the addition of some sorbents (Castaldi et al., 2005), suitable change of soil acidity (Clemente et al., 2006) or redox conditions (Groudev et al., 2010) as well as the establishment of suitable covers of the heavily contaminated sites (Komnitsas et al., 1999). The second group includes methods where the main goal is completely opposite to the previous – to create and maintain conditions, which enhance the pollutants leaching from soil horizon and their downward migration by means of draining soil solutions (Kumar and Nagendran, 2009) or pollutants` migration in the upward direction by means of their uptake and accumulation into the plant biomass (Bhargava et. al., 2012), respectively. Regardless of the chosen method, there are several key factors which preliminary evaluations determine the process efficiency at a later stage. First of all, solid phases` pollutants characterization by means of suitable extraction tests (Tessier et al., 1979), the results of which throw light on the main mobile fractions as well as stimulation of which biogeochemical process will lead to their redistribution in a suitable direction. The second key factor is acid-base soil property, which reflects on soil pH. Its value determines in large extent the size and charge, existing on the surface of soil grains (Dube et al., 2001) and if there is a need to manipulate it during application of the relevant remediation method (Harter and Naidu, 2001). The third factor concerns the chemistry of pollutant itself – valence state(s), speciation and stability of its soluble forms, etc., in depends on the soil condition. Exact evaluation of all that information allows elaborating a suitable strategy for soil remediation. Regardless of the decreasing of total concentration of pollutant and/ or its leaching in soil horizon, most methods for soil remediation are connected with changes of some basic parameters of the soil biotope. Assessing their effect towards soil biota is carried out by ecotoxicological tests which determine if there is a need for additional steps before the cleaned soil is used in agriculture.

The main aim of this study was to evaluate possibilities for *in situ* transformation of heavy metals in the soil horizon A by means of the selective dissolution of that mobile fraction in which the higher part of the pollutants are concentrated in. One of the approaches is based on the creation and maintenance of highly acidic conditions in the topsoil which initiated acidolysis and the secondary oxide minerals and leaching out of the heavy metal ions out of the topsoil. The second approach is based on the creation and maintenance of anaerobic conditions in the topsoil which initiated redoxolysis of the secondary iron oxide minerals. In that case, the heavy metal ions, which were liberated into the soil solution, were

selectively precipitated as sparingly phosphates within the soil horizon A. Both technological methods were tested in laboratory conditions by means of zero suction type lysimeters.

MATERIALS AND METHODS

The soil sample used in the experiment belonged to the grey forest soil type. The soil profile was consisted of: horizon A (0-30 cm), horizon B (31-70 cm), horizon C (71-90 cm), and a clay horizon (91-110 cm). The soil pH was determined at 1:2.5 ratio with distilled water or 1N KCl, respectively. Humus content, cation exchange capacity (CEC) and carbonate content was determined by means of suitable methods (Pansu and Gautheyrou, 2006). Elemental analysis of the digested soil sample was determined by atomic absorption spectrometry (AAS) and induced plasma spectrometry (ICP). The mobile forms of heavy metals and uranium were determined by means of a sequential extraction method (Tessier et al., 1979). The bioavailability of heavy metals and uranium was determined by means of double acid (Mehlich 1) and DTPA-test, respectively, (Nelson, et al., 1953; Lindsay and Norvell, 1978).

The soil permeability was determined by means of double ring infiltrometer method (U.S.EPA, 1991).

Soil remediation

The soil sample was treated in zero suction laboratory lysimeters. Each lysimeter was charged with 2.5 kg of topsoil. A sand layer was located beneath the soil profile which enhanced the soil solutions to drain easily. Lysimeter 1 was irrigated with solutions containing 0.10 g/l NH_4Cl, and 0.02 g/l K_2HPO_4. Elemental sulfur (0.02 %) and finely cut straws (0.02 %) were added to and mixed with the soil in Lysimeter 1. Calcium carbonate (0.16 %), hydroxyapatite (3.7 %), compost (5.5 %), and montmorilonite (4 %) were added to the soils in Lysimeter 2. Both lysimeters were irrigated with solutions containing 0.10 g/l NH_4Cl, and 0.02 g/l K_2HPO_4. The irrigation rate was 100 l/t soil per week and 200 l/t soil per week for Lysimeter 1 and Lysimeter 2, respectively. Each week the pregnant soil effluents were replaced by fresh solutions with the relevant initial composition. The leaching was carried out at varying temperatures in the range of about 15-23°C. The duration of soil remediation was 9 months for Lysimeter 1 and 18 months for Lysimeter 2, respectively.

Chemical analyses

The heavy metals' transport through the soil profile was monitored regularly by means of regular monitoring of the pregnant soil effluents collected by means of preliminary installed gravity sampling devices. The solutions were characterized by measurement of pH, Eh, acidity, dissolved organic carbon (APHA, 1995). The concentrations of heavy metals and uranium were determined after the preliminary digestion of dissolved organic compounds by means of

705 UV Digester (Metrohm). The heavy metals were analyzed by means of ICP spectrophotometry. Uranium concentration was measured photometrically using the Arsenazo III reagent (Savvin, 1961).

Lime requirement

Lime requirement of the topsoil was determined by means of Adams-Evans buffer method (Adams and Evans, 1962).

Ecotoxicity analyses

The ecotoxicity analyses were carried out with the non-treated soil sample of horizon A as well as samples of the topsoil which have been remediated at the relevant conditions. The soil toxicity towards oats (*Avena sativa*) was determined in accordance with the range-finding test (OECD, 1984) with the purpose to establish dose-response relationship to the plant species towards the tested soil sample. The test concentrations of soil sample were in the range of 1-100 % (weight) and the rest milieu for plant growth was composted biomass. Each pot was sown with ten seeds. Three replicates were used for each test concentration as well as for controls of each species. In the control the seeds were sown in composted biomass (pH (H_2O) 5.9-6.1). The test was carried out in a greenhouse at temperatures 16–22°C, and precise control on the duration of photoperiod (16 hours) and the soil humidity (maintained by means of distilled water). The test's duration was 30 days.

The soil toxicity towards earthworm (*Lumbricus terrestris*) was carried out with a synchronized population, which was cultivated preliminary for 1 year at laboratory conditions in grey forest soil. The toxicity of the soil samples was determined by range-finding and definitive test (U.S.EPA, 1996) which were carried out in plastic boxes with volume of 1.0 l. The test concentrations of soil sample were in the range of 1-100 % (weight) and the rest milieu was brown forest soil. Three replicates were used for each test concentration with ten worms with similar length added to each. Ten worms were added to the control too, which consisted of brown forest soil only. The duration of the test was 30 days. The worms' survival and signs of their activity was determined at the end of the toxicity test.

The data from all replicates of each test concentration of the relevant soil sample to the relevant species were statistically assessed by means of determination of the average values and standard deviation. The main ecotoxicity parameters-No Observed Effect Concentration (NOEC), the Lowest Observed Effect Concentration (LOEC), LC_{50} and LC_{100} were determined by processing of experimental data by means of Shapiro Wilk`s test and the Probit method, respectively (U.S.EPA, 1994).

Study site

The uranium mine Curilo, located in Western Bulgaria for a period of four decades was a site for intensive mining activities, including both open-pit and underground techniques as well as *in situ* leaching of uranium by means of

injection of sulfuric acid solutions. These activities ceased in 1990, however, the fractured ore body and the dumps consisting of mining wastes are still an intensive source of acid drainage waters. The generation of such waters is a result mainly of bacterial oxidation of pyrite, other sulphides as well as uranium-bearing minerals presented in the mining wastes. A natural collector of waters, which have been infiltrated through the mine, is the acidic lake. The total volume of the lake is in a range of 4500-5500 m^3 and waters are characterized with acidic pH and higher concentration of dissolved iron, non-ferrous metals, and uranium. Jarosite $(X.Fe_2(SO_4)_3.(OH)_6)$ and ferric iron hydroxides $(Fe(OH)_3)$ are the iron oxide minerals, which are formed as a result of the bacterial oxidation of ferrous iron to ferric state by means of chemolithotrophic iron-oxidizing bacteria (*Leptospirillum ferrooxidans, Acidithiobacillus ferrooxidans*) followed by partial hydrolysis of ferric iron. However, higher part of the iron minerals were dispersed into the water column due to the higher concentration of dissolved solids and higher specific density of the lake`s waters. The acid drainage waters flow out of the acidic lake and run into the local Teina river. Despite the dilution effect, the concentrations of iron, non-ferrous metals, uranium and water pH were above the permissible levels of waters intended to use in industry as well as agriculture. The soils situated near to the river banks are regularly flooded by the AMD-impacted waters which lead to their pollution and degradation.

Grey forest soil is the soil type in the studied area which had been formed under the regime of higher rainfalls' rate and lower annual temperature (Koinov et al., 1998). As a result, the depth of humus layer is lower in comparison to the lowland soils, as well as the typical texture differentiation within the soil profile is easily distinguished with the formation of a clayish illuvial horizon into the soil depth. The depth of the soil profile is not high (0-80 cm) and three soil horizons are distinguished in it – soil horizon A (0-15 cm), horizon B (16-40 cm), and horizon C (41-70cm), respectively. The humus content of the topsoil is usually below 3%, the content of carbonates, iron and aluminium oxides is increased into the soil depth which explain the slightly acidic soil pH of horizon A. That differentiation explained the lower water permeability of below-lying soil horizons.

Bulgarian guidelines for soil pollution with heavy metals are based on the value of soil pH and the total concentration of the element in the topsoil of relevant soil type (Guideline № 3, 2008). As the standard applied in other countries (CCME, 2006), it considers the different degree of pollution and regulates different thresholds-background, target, maximum admissible, and intervention concentrations, which values depend on the relevant land use (agricultural, residential, or industrial).

RESULTS AND DISCUSSIONS

The studied soil plot was heavily polluted with heavy metals as well as uranium. For example, the contents of copper and lead in the topsoil, which are two of the most toxic heavy metals, were 2.6 and 1.1 times higher than the relevant permissible levels for soils in Bulgaria (Table 1). The content of uranium was 1.8 times higher than the relevant permissible concentrations for soils with neutral soil pH. The higher concentrations of these pollutants as well as highly acidic soil pH were the reasons the studied soil plot to be classified as highly risky towards the environment and human health and the present study to be carried out.

Table 1. Data about the chemical content and some properties of the soil horizons of the studied soil.

Index	Soil horizon	
	A (0-15 cm)	B (16-40 cm)
SiO_2, %	71.1	67.5
Al_2O_3, %	17.2	18.8
Fe_2O_3, %	4.7	6.8
K_2O, %	2.1	2.2
P_2O_5, %	0.15	0.12
N_{total}, %	0.07	0.04
Carbonates, %	< 0.2	0.7
Humus, %	0.9	0.3
Pb, mg/kg	102	28
Cu, mg/kg	210	112
Zn, mg/kg	172	96
U, mg/kg	18.6	6.3
pH (H_2O)	3.75	3.71

The sequential extraction test and tests for bioavailability revealed the distribution of the soil contaminants amongst the main mobile fractions and at which conditions the heavy metals could be highly available for soil microflora and plants. For example, the reducible fraction was the main mobile fraction in which was concentrated about 50% of the relevant amount of lead, copper, zinc, and uranium in soil horizon A (Table 2). That fraction presented the native iron oxide, which is typical for soils (hematite, magnetite, etc.,) as well as the ferric iron oxyhydroxides (jarosite, goethite, shwartmanite, etc.,) which were deposited in the topsoil as a result of the pollution by acid mine drainage. The oxidisable fraction was the second important fraction where between 22-50% of

the pollutants were presented. Having in mind the very low content of organic compounds in the polluted soil plot and that the Kurilo area is geochemically enriched, the oxidisable fraction of heavy metals and uranium in the soil horizon A were presented probably by the relevant sulfide minerals and uraninite, respectively. Regarding the easily leachable fractions of the soil pollutants - exchangeable and carbonate – between 0.4-5.0% of each pollutant were presented on each of it. However, it was a significant difference in the way of distribution of the soil pollutants amongst these mobile fractions. Because of highly acidic soil pH, the freshly formed ferric iron oxyhydroxides in the topsoil were characterized with positive surface charge which was neutralized by means of attraction and adsorption of anions from the soil solution. That process is the ground of the so-called specific adsorption of non-ferrous metals in soils. For soils with slightly acidic to neutral soil pH, the carbonates are minerals on which surface the process is carried out. When the carbonates are dissolved in the environment with higher acidity, the surface of ferric iron oxyhydroxides has started to govern the process of selective adsorption of heavy metals and uranium. For that reason, it will be more correct for the carbonate mobile fraction of the pollutants to be renamed to specifically adsorbed heavy metals. Amongst the soil pollutants, the content of uranium in that fraction was the highest – 5.0 percent. It is well-known that uranium migrates in the environment by means of uranyl- sulfate and uranyl-carbonate complexes, which are typical at acidic and neutral-alkaline conditions, respectively. It is interesting to note that specifically adsorbed heavy metals on the surface of ferric iron oxyhydroxides could be transformed into reducible fraction for a relatively short period of time when the freshly formed amorphous ferric iron hydroxide are deposited on the already existing.

Table 2. Data about the main mobile fractions of lead, copper, zinc and uranium in soil horizon A before the soil remediation.

Mobile fraction	Heavy metals							
	Pb		Cu		Zn		U	
	mg/kg	%	mg/kg	%	mg/kg	%	mg/kg	%
Exchangeable	1.2	1.2	4.8	2.3	8.6	5.0	0.3	1.7
Carbonate	0.4	0.4	8.0	3.8	2.2	1.3	0.5	2.7
Reducible	50.4	49.4	103.9	49.5	86.9	50.5	7.5	40.3
Oxidisable	23.3	22.8	63.4	30.2	36.5	21.2	9.3	49.8
Inert	26.7	26.2	29.8	14.2	37.8	22.0	1.0	5.5
Total	102	100	210	100	172	100	18.6	100

Regardless of that distribution of the heavy metals among the mobile fractions, their mobility could be dramatically changed in the presence of a suitable complexing agent at relevant soil pH (Table 3). For example, even at

neutral pH and in the presence of a polycarboxylic organic acid, the mobility of copper and lead was very high. It meant that as a result of typical soil amelioration as neutralization and composting, the studied soil plot still be highly toxic to soil biota. The mobility of zinc in the topsoil was different. Zinc as an element formed stable complexes with inorganic complexing agents (chlorides, nitrates, etc.,). For that reason, the higher mobility of the element was determined at highly acidic conditions. Ecotoxicity of the contaminated soils towards soil biota was determined by the negative changes in the physical structure of biotope, which had an effect on the water-holding capacity, air permeability, chemical properties (pH, pollutants, etc.) which in dependence on the magnitude of changes determined the acute or the relevant chronic effect of toxicity. For that reason, the application of species with higher sensitivity towards the relevant negative impact is highly advisable to be used in ecotoxicological studies. For that reason, oats (*Avena sativa*) and earthworms (*Lumbricus terrestris*) were chosen as suitable species to which ecotoxicity of the polluted soil horizon A was characterized. So, the toxicity of the topsoil was 8 and 20 toxicity units towards oats and earthworms, respectively, which mean that one-half of test organisms die during the relevant test, if the portion of the polluted soil during the test was above 12.5 % and 5 %, respectively.

Table 3. Data about the bioavailability of lead, copper, zinc and uranium in soil horizon A before the soil remediation.

Test	Heavy metals							
	Pb		Cu		Zn		U	
	mg/kg	%	mg/kg	%	mg/kg	%	mg/kg	%
DTPA	17.4	17.1	33.2	15.8	6.4	3.7	0.5	2.9
H_2SO_4 + HCl	1.4	1.4	2.3	1.1	55.5	32.3	0.04	0.2

Elemental sulfur is an ameliorant widely used in agriculture for reclamation of salt-affected soil, containing carbonates in the topsoil. Sulfur-oxidizing chemolithrophic bacteria (*Thiobacillus thioparus, Th. neapolitanus*) growing at neutral soil pH are responsible for the generation of sulfuric acid into the soil by means of oxidation of the presented reducible forms of inorganic sulfur. In a similar manner, elemental sulfur was used as an ameliorant for biotechnological remediation of AMD-affected soils. In that case, the iron oxyhydrooxides, deposited in the topsoil, were the buffer systems responsible for the consumption of sulfuric acid generated as a result of bacterial oxidation of elemental sulfur. That process was connected not only with the consumption of the hydrogen ion but also with the dissolution of ferric oxide minerals with amorphous structure and the release of the non-ferrous metals and uranium which had been specifically adsorbed on the surface or capsulated in their crystal lattice. That is

a second order process which is strongly dependent on the surface area of the ferric oxyhydroxides. For that reason, it was needed to provide longer time for reaction between them and the generated sulfuric acid. It was achieved by means of addition of finely cut straw as an ameliorant in the very beginning of the soil remediation. Its role was to enhance the growth and activity of soil fungi, which play the main role in degradation of organic compounds in acidic soils. Soil fungi and sulfur-oxidizing chemolithotrophic bacteria are aerobes and used the molecular oxygen as a final acceptor of electrons in their microbial metabolism. So, competition between fungi and sulfur-oxidizing bacteria has been aroused towards molecular oxygen when its concentration in the soil was limited. For example, the number of both microbial groups was in the similar range in the topsoil during the method application (Table 4). As a result of that, the bacterial oxidation of elemental sulfur and generation of sulfuric acid into the soil's solution was steadily decreased, which determined the variation of pH of the pregnant soil effluents to vary in a narrow range during the soil remediation (Table 5). Another advantage of fungal metabolism was the generation and secretion of full range of organic acids (citric, oxalic, maleic, etc.) into the soil biotope. All organic acids are characterized by excellent complexing properties, especially to heavy metals (Sterflinger, 2000). So, the highest concentration of heavy metals and uranium in the pregnant soil effluents was measured between 3-6 months since the start of method application in Lysimeter 1 (Table 5).

Table 4. Data about the microflora of soil horizon A (0 – 15 cm) of grey forest soil before and during the soil remediation.

Microorganisms	Before soil remediation	Lysimeter	
		1	2
S°-oxidizing chemolithotrophs	$10^2 – 10^3$	$10^6 – 10^7$	$< 10^1$
Nitrifying bacteria	$10^1 - 10^2$	$10^1 - 10^3$	$10^4 – 10^5$
Fe^{2+}-oxidizing bacteria	$10^2 – 10^3$	$10^2 – 10^3$	$10^1 - 10^2$
Aerobic heterotrophic bacteria	$10^3 - 10^4$	$10^3 – 10^4$	$10^5 - 10^6$
Fungi	$10^3 - 10^4$	$10^5 – 10^6$	$10^3 – 10^5$
Anaerobic heterotrophic bacteria	$10^3 - 10^5$	$10^4 – 10^6$	$10^6 – 10^8$
Cellulose-degrading bacteria	$10^1 - 10^2$	$10^2 – 10^3$	$10^4 – 10^5$
Denitrifying bacteria	$10^1 – 10^2$	$10^2 – 10^3$	$10^2 – 10^3$
Fe^{3+}-reducing bacteria	$10^2 - 10^4$	$10^2 - 10^3$	$10^5 - 10^6$
Sulfate-reducing bacteria	$10^2 – 10^3$	$10^3 – 10^4$	$10^3 – 10^5$

Table 5. Characteristic of the pregnant effluents generated from the soil horizon A between 3-6 months at the relevant conditions of soil remediation.

Parameter	Before soil remediation	Lysimeter	
		1	2
pH	3.95	2.38-3.35	5.33 – 6.25
Eh, mV	(+340)	(+480)-(+545)	(+70) – (+120)
Acidity, mmol/l	3.8	13.5-68.3	0.2 – 0.5
Dissolved organic carbon, mg/l	< 5.0	18-35	27-63
Pb, mg/l	0.02	0.33-0.5	< 0.03-0.2
Zn, mg/l	1.3	3.9-6.4	0.2-0.5
Cu, mg/l	1.5	10.3-16.8	0.1-0.4
U, mg/l	0.25	0.85-1.2	0.02-0.04
Fe, mg/l	9.6	342-420	2.3-6.4
Mn, mg/l	23.3	35-50	9.5-14.3
Ca, mg/l	58-70	88-125	112-165
Mg, mg/l	25-31	38-52	49-65
Al, mg/l	18.4	218-335	1.8-7.2
SO_4^{2-}, mg/l	1030	540-810	75-132

After 9 months of soil remediation at such experimental conditions, the concentrations of lead, copper, and uranium were decreased by 23.2%, 63.1%, and 44.6%, respectively. Also, 40.5% of the initial content of zinc was leached out of soil horizon A (Table 6). The residual concentration of all heavy metals in the topsoil was below the relevant Maximum Admissable Concentration (MAC) for soil. The residual concentration of uranium was very close to the relevant MAC value.

The higher leaching of the heavy metals by means of acidolysis followed by soil neutralization determined the significant reduction of the soil toxicity towards used test species. For example, the residual toxicity of the treated soil towards oats was 2 toxicity units, which determined 75% of reduction (Table 7). The toxicity towards earthworms was reduced by 87.5%. The residual toxicity of the soil remediated at conditions of Lysimeter 1 probably was determined by the deep structural changes of the mineral phase of the soil which were carried out as a result of the method application.

Table 6. Data about the content of heavy metals at the end of soil remediation at the relevant experimental conditions.

Heavy metals	Before soil remediation	Lysimeter		Maximum Admissible Concentration for agricultural soil with pH>7.4
		1	2	
Pb, mg/kg	102	78.3	98	90
Zn, mg/kg	210	125	192	200
Cu, mg/kg	172	63.5	165	80
U, mg/kg	18.6	10.3	14.8	10
pH (H_2O)	3.75	6.65	6.18	-

Table 7. Data about the ecotoxicity of the soil horizon A to oats and earthworm before and after the soil remediation.

Species	Ecotoxicity parameter	Before soil remediation	After soil remediation	
			Lysimeter 1	Lysimeter 2
Oats (*Avena sativa*)	LC_{50}	12.5	50	-
	Toxicity units	8	2	-
	Reduction of toxicity, %	-	75	100
Earthworm (*Lumbricus terrestris*)	LC_{50}	5	40	-
	Toxicity units	20	2.5	-
	Reduction of toxicity, %	-	87.5	100

Apart from acidolysis, the ferric iron hydroxides could be dissolved also by means of redoxolysis process. In the ecosystems that process is carried out by iron-reducing bacteria in the anaerobic zones of biotope enriched with organic compounds. The iron-reduction is a trait widely used amongst bacteria, and it is displayed when the concentration of alternative acceptors of electrons (such as oxygen, nitrate, and nitrite) is already exhausted. That process is alkaline generating, and it plays a crucial role in maintaining constant pH in river bed sediments as well as the upper soil horizons (Marshner and Noble, 2000). The process of iron reduction in the soil horizon A was stimulated by means of carbonates, compost, and montmorilonite addition. The carbonates were added in an amount for partial neutralization and to increase the soil pH to 4.2-4.3 which was sufficient to start the process of reintroduction of all heterotrophic microorganisms as a sole part of the soil microflora when organic carbon in the soil was increased. The presence of higher amount of plant compost plus montmorilonite enhanced establishment and maintenance of anaerobic conditions

in the topsoil (Table 5). In such conditions, the iron reduction was dominated process due to the absence of higher concentration of alternative acceptors of electrons (Table 4).

In the very beginning of the soil remediation applied in Lysimeter 2, hydroxyapatite was also added to the soil, which made possible to prevent the leaching of already released heavy metals and uranium ions out of the soil horizon A. The advantage of hydroxyapatite as ameliorant is that it allowed their selective precipitation to be carried out in an environment characterized by acidic pH, higher concentration of inorganic and organic ligands, as well as higher concentrations of base and other non-ferrous metals (Chen et al., 1997). For that reason, hydroxyapatite is highly used for selective precipitation of pollutants as lead, copper, nickel, uranium, etc., For example, lead and copper are precipitated as pyrromorphite ($Pb_{10}(PO)_4(OH)_2$) and copper phosphate ($Cu_3(PO_4)_2$), respectively, at such conditions. Pyrromorphite and its derivatives (chlorpyrromorphite, fluorpyrromorphite, etc.,) are the most resistant minerals of lead (Ma and Rao, 1997). During the soil treatment, the concentrations of heavy metals and uranium in the pregnant soil effluents were decreased more than 10 times in comparison to the concentrations measured in the effluents of non-remediated soil (Table 5).

After 18 months of soil remediation at such experimental conditions, the concentration of lead and copper was decreased negligible (3.9% for lead and 4.1 % for copper, respectively) in comparison to their initial content in soil horizon A (Table 6). Apart from the applied conditions which enhanced the pollutants' precipitation, around 20% of initially presented uranium was leached out from the topsoil. However, the residual concentration of lead, copper, and uranium in the topsoil was above the relevant Maximum Admissible Concentration (MAC) for soils.

Regardless of the insignificant leaching of the heavy metals from soil horizon A in comparison to their leaching that was achieved as a result of soil remediation applied in Lysimeter 1, the acute toxicity of the treated soil from Lysimeter 2 towards oats and earthworms was removed completely (Table 7). It revealed that the toxicity of heavy metals towards organisms is determined by the amount of their bioavailable forms instead of their total content into biotope.

CONCLUSIONS

The long-term local impact of the acid mine drainage, generated as a result of bacterial oxidation of sulfides, on soils lead to exhaustion of all carbonates, very acidic soil (pH (H_2O)~3,2), and higher concentrations of heavy metals in the soil horizon A, presented mainly as reducible mobile fraction.

Acidolysis of amorphous ferric oxyhydroxides was carried out for a relatively short period of time into the soil as a result of *in situ* generated sulfuric acid

due to bacterial oxidation of added S° by means of acidophilic sulfur-oxidizing chemolithotrophic bacteria. As a result of that process, the heavy metal ions, which have been capsulated in their lattice, was liberated and their intensive leaching out from soil horizon A by means drainage soil effluents was possible.

The partial neutralization of the soil acidity to pH (H_2O)~4.3, followed by the creation and maintenance of anaerobic conditions in the soil horizon A allowed amorphous ferric oxyhydroxides to be reduced as a result of the growth of the iron reducing bacteria. It was connected also with the release of the heavy metal ions, which have been capsulated in the relevant crystal lattice to that moment, and their selective precipitation in the soil horizon as the relevant sparingly soluble phosphates.

The results of the ecotoxicity studies revealed that the acute toxicity of the soil, remediated by means of selective precipitation of the heavy metals as sparingly soluble phosphates, towards oats (*Avena sativa*) and earthworm (*Lumbricus terrestris*) was reduced completely. Despite the considerably lower content of the heavy metals in the soil remediated by acidolysis it was residual acute toxicity to test organisms. The additional destruction of the soil matrix as a result of generation of sulfuric acid probably was the main reason for that result.

REFERENCES

1. Adams, F., Evans, C. E. 1962. A rapid method for measuring lime requirement of red-yellow podzolic soils. *Soil Sci. Soc. Amer. Proc*, 26: 355-357.
2. APHA., 1995. Standard methods for the examination of water and wastewater. American Public Health Association, Washington, D.C.
3. Bhargava, A., Carmona, F.F., Bhargava, M., Shrivastava, S., 2012. Approaches for enhanced phytoextraction of heavy metals. *Journal of Environmental Management*, 105: 103-120.
4. Castaldi, P., Santona, L., Melis, P., 2005. Heavy metal immobilization by chemical amendments in a polluted soil and influence on white lupin growth. *Chemosphere*, 60: 365-371.
5. CCME (Canadian Council of Ministers for the Environment), Canadian soil quality guidelines for the protection of environmental and human health, update 6.02, Publication no 1299, November 2006, ISBN 1-896997-34-1.
6. Chen, X., Wright, J.V., Conca, J.L., Peurrung, L.M. 1997. Effects of pH on heavy metals sorption on mineral apatite. *Environmental Science & Technology*, 31: 624-631.
7. Clemente, R., Almela, C., Brenal., M.P. 2006. A remediation strategy based on active phytoremedation followed by natural attenuation in a soil contaminated by pyrite waste. *Environmental Pollution*, 143: 397 – 406.
8. Dube, A., Zbytniewski, R., Kowalkowski, T., Cukrowska, E., Buszewski, B. 2001. Adsorption and migration of heavy metals in soils. *Polish Journal of Environmental Studies*, 10: 1-10.

9. Groudev, S., Spasova, I., Nicolova, M., Georgiev, P. 2010. In situ bioremediation of contaminated soil in uranium deposits. *Hydrometallurgy*, 104: 518 – 523.

10. Guideline №3. Permissible contents of harmful substances in the Bulgarian soils. *An Official Gazette* 71, 12th of August, 2008.

11. Harter, R., Naidu, R., 2001. An assessment of environmental and solution parameter impact on trace-metal sorption by soils. *Soil Science Society of America Journal*, 65: 597-612.

12. Komnitsas, K., Lazar, I., Petrisor, I. 1999. Application of a vegetative cover on phosphogypsum stacks. *Minerals Engineering*, 12: 175-185.

13. Koinov, V.A., Kabakchiev, I., Boneva, K. 1998. Zemizdat, Sofia, 323pp.

14. Kumar, R.N., Nagendran, R., 2009. Fractionation behavior of heavy metals in soil during bioleaching with *Acidithiobacillus thiooxidans*. *Journal of Hazardous Materials*, 169: 1119–1126.

15. Lindsay, W.L., Norvell, W.A., 1978. Development of DTPA soil test for zinc, iron, manganese, and copper. *Soil Science Society of American Journal*, 42: 421-428.

16. Ma, Q.Y., Rao, G.N. 1997. Effects of phosphate rocks on sequential chemical extraction of lead in contaminated soils. *Journal of Environmental Quality*, 26: 788-794.

17. Marscher, B., Noble, A.D. 2000. Chemical and biological processes leading to the neutralization of acidity in soil incubated with litter matters. *Soil Biology and Biochemsitry*, 32: 805-813.

18. Nelson, W. L., Mehlich, A., Winters, E. 1953. The development, evaluation, and use of soil tests for phosphorus availability. *Agronomy*, 4:153-158.

19. OECD., 1984. Guideline for testing of chemicals. 208. Terrestrial plant test: Vegetative Vigour Test 3-7.

20. Pansu, M., Gautheyrou, J. 2006. Handbook of Soil Analysis. Mineralogical, Organic and Inorganic Methods. Springer Verlag, Berlin Haidelberg.

21. Savvin, S.B., 1961. Analytical use of arsenazo III: determination of thorium, zirconium, uranium and rare earth elements. *Talanta*, 6: 673 – 685.

22. Sterflinger, K. 2000. Fungi as geologic agents. *Geomicrobiology Journal*, 17: 97-124.

23. Tessier, A., Campbell P.G.C., Bisson, M., 1979. Sequential extraction procedure for speciation of particulate trace metals. *Analytical Chemistry*, 51: 844 - 851.

24. U.S.EPA., 1991. Description and Sampling of contaminated soils-Field Pocket Guide. EPA/625/12-91/002.

25. U.S.EPA., 1994. Methods for assessing the toxicity of sediment-associated contaminants with estuarine and marine amphipods. EPA 600/R-94/025.

26. U.S.EPA., 1996. Ecological effects test guidelines. OPPTS 850.6200. Earthworm subchronic toxicity test. EPA 712-C-96-167.