THE CAPACITY OF MICROBIAL ISOLATES FROM NUCLEAR WASTE REPOSITORY SOIL TO UPTAKE TOXIC METALS

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Abstract. This paper reveals the ability of microbial communities isolated from a nuclear waste repository soil to withstand high radiation background, high concentrations of toxic heavy metals, and to reduce the concentration of certain heavy metals in the culture medium. After gamma irradiation, 12 resistant bacterial strains were isolated. These bacteria exhibited increased abilities in removing toxic concentrations of ^{63}Cu, ^{59}Co, and ^{133}Cs from the culture medium.

Key words: radioresistant microorganisms, bioremediation, soil microorganisms.

1. INTRODUCTION

Accumulation of heavy metals, radionuclides and different chemical compounds (organic and inorganic) into the environments (soil and water) is a factor of great concern worldwide, due to their long-lasting effects on the ecosystem. Many metals exhibit toxic (cytotoxic, carcinogenic, and mutagenic) effects even at low concentrations (arsenic, copper, lead, mercury, cadmium, etc.) [1]. Environmental pollution is a perpetual problem that persists and affects human health. To decontaminate these polluted environments, several approaches can be applied. Chemical precipitation, membrane technologies, ion exchange, oxidation/reduction are some techniques that are used to reduce the concentrations of the contaminant. Even if these methods have some efficiency in removing the contaminant, they became ineffective when the concentration of the heavy metal is very low [2], while many heavy metal salts are highly water-soluble, thus they cannot be separated by using chemical and physical methods.

A promising approach is the use of microorganisms and plants in heavy metal and radionuclides decontamination processes [3, 4].

Every microbial community is a highly adapted system where different types of microorganisms influence each other by their byproducts, as well as by intense competition for ecosystem resources (carbon and nitrogen). Their metabolism is perfectly adjusted, supporting the propagation of selected species and even small variation in the environment composition and physical parameters can dramatically influence the microbial community composition. Very restrictive environments,

contaminated with different types of pollutants (heavy metals, organic/inorganic compounds, radionuclides) present highly specialized microbial communities able to use the contaminants in internal metabolic processes [5, 6–8]. The response of microbial communities to heavy metals and radionuclides is a complex process that depends on several aspects such as the concentration and availability of the metal or radionuclide, the nature of the environment and the microbial species [9].

The microorganisms can actively (through bioaccumulation) and passively (through adsorption) uptake the heavy metals and radionuclides from environments, both processes being influenced by some chemical factors such as pH, temperature, O_2 concentration [10]. The microbial cell walls are organized by polysaccharides, lipids, and proteins that offer a plethora of functional groups (carboxylate, hydroxyl, amino, phosphate, alcohol, ester, sulfhydryl, thioether, thiol) which can bind heavy metal ions.

The viable microbial concentration in soil samples was around 10^6–10^7 CFU/g of soil. We must keep in mind that these are only the cultivable soil microorganisms, a small fraction of its total microbial count; many other species are plausible to co-exist and not multiply under the applied cultivation method. The ability of these communities to withstand high gamma radiation intensity was challenged. Since the analyzed soil samples have a high natural radioactivity (Table 1) we expect that bacterial community to have also an increased radiation resistance. The Biolog Ecoplate® was used to analyze the microbial community metabolic profile. Although the soil samples come from a very special environment with low temperature and low organic load, they show evidence of a rich microbial diversity.

2. MATERIALS AND METHODS

2.1. STUDY SITE

The soil samples were collected from the National Radioactive Waste Repository (NRWR) Baiţa, Bihor County, Romania. The repository is an exhausted underground uranium mine in the Apuseni Mountains, the western part of the Carpathian Mountains, at an altitude of 840 m. This repository was commissioned in 1986, following a widening of the galleries and execution of a drainage system for the collection of some potentially contaminated water in a collection tank. The rock formation in the repository area are meta-sandstones, phyllite, and basalt. The repository is designed to dispose of about 5000 m^3 of low and intermediate-level short-lived waste, institutional radioactive waste from industrial, medical, and research activity like ^{51}Cr, ^{60}Co, ^{90}Sr, ^{90}Y, ^{123}I, ^{137}Cs, ^{192}Ir [11]. Even if the mine is depleted, the rocks still exhibit an important level of radioactivity due to the radon, the remaining trace of uranium ore, as well as the natural K and Th that are components of the repository background rocks.

The structure and granularity of the soil samples were similar, with only small differences. Macroscopically, all three samples were a combination of fine and coarse sand with roughly the same humidity, ranging between 75%–83% rH.

2.2. SOIL SAMPLING

Soil samples were collected from three soil radioactivity hot spots inside Baita (NRWR). The samples have uneven composition, mainly consisting of soil, sand, small rocks. They were taken from the upper layer of soil, using sterile instruments, labeled accordingly (S6; S11; S12), weighted, and stored at 4°C.

2.3. MICROBIAL COUNT AND RADIATION-RESISTANT BACTERIA SELECTION

Both quantifications of total bacteria and selection of bacteria were performed using standard microbiological methods. For the quantification of soil culturable microbiota, 10 g of soil was hydrated using sterile water. The resulting soil suspension was incubated for 3 h at 25°C to revive the microorganisms. After incubation, seven tenfold dilutions were performed, and 1 ml suspension was filtered through 0.45 μm pore size filters (Merck®).

The filters were inoculated onto R2 agar plates (Merck®) and the resulting inoculated agar plates were incubated at 25°C for 5 days. Viable culturable microorganisms were quantified by colony counting.

For the isolation of radiation-resistant bacteria, 10 g soil samples were first dried with ventilation at 30°C for 48 h, then irradiated using a ^{60}Co gamma research irradiator (model GC-5000 manufactured by B.R.I.T., India) at an average dose of 4 kGy, with a dose rate of 4 kGy/h. The irradiation of the same sample in 2 forms (dried and wet) was realized in order to observe the influences of free radicals, resulted from gama irradiation of their growth liquid medium, on bacterial viability. Both experiments were run simultaneously, in three distinct replicates for every soil sample.

2.4. COMMUNITY-LEVEL PHYSIOLOGICAL PROFILING

The metabolic profile of each microbial community present in the three soil samples was assessed by using Biolog Ecoplate™ (Biolog Inc., Hayward, CA, USA) [12]. The Ecoplate has a special design, it consists of 96 wells, containing 31 different carbon sources and a blank well (in triplicate), giving the possibility to obtain statistical insights. Every carbon source is mixed with tetrazolium violet redox dye which changes its color to a purple shade if the carbon source from the well is reduced by the microorganism [13]. While bacterial communities utilize carbon sources, the tetrazolium salt is reduced and forms a purple color. Therefore, the microbial communities will exhibit a characteristic metabolic fingerprint (color

pattern) representing the metabolic properties of the community. The Ecoplate carbon sources are divided into five substrate groups: amino-acids, amines, amides, carbohydrates, carboxylic and acetic acids, and polymers. The testing protocol was performed as follows: 1 g of soil was hydrated with 99 ml of sterile water and incubated in a shaking incubator for 2 h at 25°C. Aliquots of 0.25 ml of the soil suspensions were then inoculated in every well and the plates were incubated at 25°C for 7 days. The absorbance dynamic of each well was read at 595 nm, at 2 h intervals, using a SpectraMax i3 reader (Molecular Devices) controlled by SoftMax Pro 7.1 software (Molecular Devices).

The average well color development (AWCD) of the solution in microplate wells was used to represent soil microbial metabolic activities. The AWCD was calculated using the following equation: $\text{AWCD} = \frac{\sum C_i - R_i}{n}$, where C_i is the OD mean value of each well with the same culture media, R_i is the OD mean value of the blank well, n is the number of the Biolog EcoPlate™ well ($n = 31$) [14, 15].

Functional and genetic diversity of soil microbial community was evaluated using the Shannon index [15].

2.5. HIGH-RESOLUTION GAMMA SPECTROMETRY AND ICP-MS ANALYSIS OF SOIL SAMPLES

To have a better insight into the soil samples, high-resolution gamma spectrometry and ICP-MS analyses were performed using standard protocols. The radiometric determinations were made using a 150 g powder sample and SARPAGAN boxes. Before the radiometric determinations, the powder soil sample was sealed in the SARPAGAN boxes and stored for 28 days to reach the radioactive equilibrium. To calculate the activity concentrations of ^{40}K, ^{232}Th, ^{238}U radionuclides, a CANBERRA system was used, consisting of an n-type 0.6 mm epoxy carbon HPGe detector with ISOXCALL characterization, DSA 1000 multichannel analyzer, and a 747-lead shield. The detector has a relative efficiency of 40% and the resolutions of 1.96 keV and 0.89 keV at ^{60}Co 1332 keV and ^{57}Co 122 keV spectral lines, respectively. The CANBERRA LabSOCS software was used to determine the efficiency calibration, self-absorption, and coincidence summing correction, while GENIE 2000 was used for data acquisition and processing. The background contribution was obtained as the average of three independent measurements, each lasting for 72 K seconds.

2.6. THE REDUCTION OF TOXIC ELEMENTS DISSOLVED IN LIQUID CULTURE MEDIUM USING ISOLATED RADIORESISTANT MICROORGANISMS

Twelve strains were selected that were able to develop distinct colonies after gamma irradiation. The capacity of selected radioresistant isolated microorganisms

to reduce toxic compounds from culture mediums was tested. To test their response to toxic compounds, 100 ml of R2 broth culture medium was supplemented with copper, cesium, and cobalt at a concentration of 1 mM and inoculated individually with pure isolates. The cultures were incubated for 5 days at 25°C in a shaking incubator at 100 rpm. The concentration dynamic of each toxic compound was analyzed using ICP MS after entire biomass collection by filtration from culture broth, using a 0.22 µm pore size membrane (Millex®, Millipore).

3. RESULTS

3.1. HIGH-RESOLUTION GAMMA SPECTROMETRY AND ICP-MS OF SOIL SAMPLES

The radiometric measurements show a high dose rate background of these soil samples, due to their contaminant elements as a normal background, the dose rate is around 0.100 µSv/h (Table 1). The concentrations of the main contaminants and their radionuclides were determined using high-resolution gamma spectrometry and the results are represented in Fig. 1; increased concentrations of K, U, and Th were also confirmed by ICP-MS (Fig. 2).

Table 1

Soil natural dose rate as measured after sampling

Soil sample	Sample weight (g)	Sample dose rate (µSv/h)
No. 6	120	2.30
No. 11	86	1.94
No. 12	104	1.78

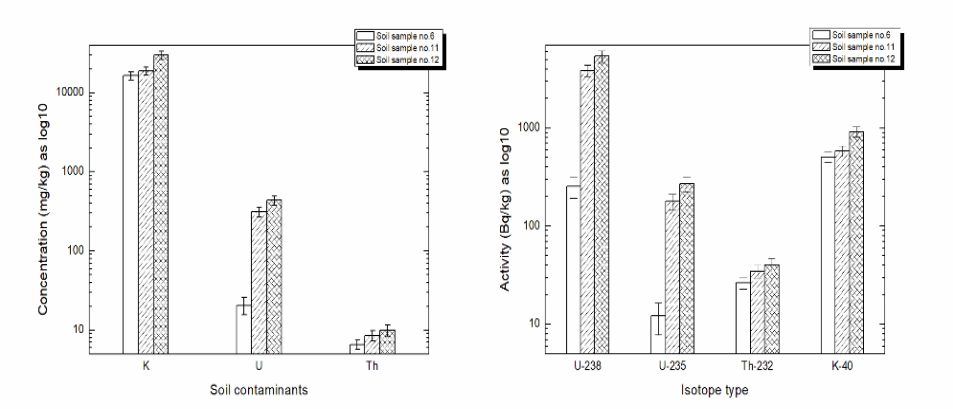

Fig. 1 – Soil contaminants (K, U, Th) concentration (left) and radionuclides activity (right) determined using high-resolution gamma spectrometry.

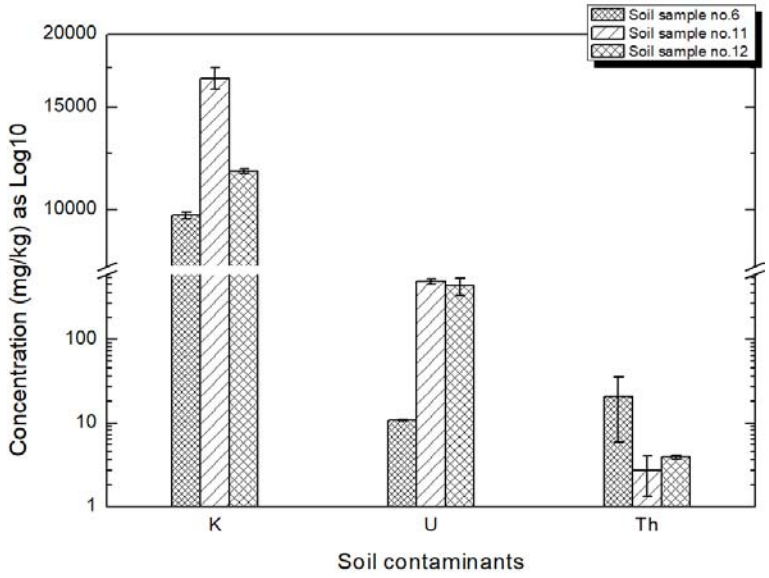

Fig. 2 – Soil contaminants (K, U, Th) concentration determined using ICP-MS.

3.2. MICROBIAL QUANTIFICATION AND ISOLATION OF RADIATION-RESISTANT BACTERIA FROM SOIL SAMPLES

After irradiation, we obtain a 2-fold microbial decrease for soil sample no. 6, a 3-fold microbial decrease for no. 12, and a 4-fold decrease for sample no. 11, respectively. The microbial community from soil sample no. 6 exhibits the highest radioresistence and this correlates with the highest radioactivity background of 2.30 μSv/h of the soil sample. From these irradiated soils, 12 bacterial strains were isolated based on their growth characteristics and subjected furthermore to a bioremediation experiment. As such, these microorganisms, mainly belonging to the *Bacillus* genus, exhibited outstanding resistance to gamma irradiation, managing to withstand a radiation dose of 4 kGy, at a dose rate of 4 kGy/h, this being a direct adaptation to the environment. Furthermore, these microorganisms were challenged to grow and reduce toxic elements (Cu, Cs, Co) dissolved in their culture medium.

3.3. MICROBIAL COMMUNITY METABOLIC PROFILE

The AWCD for all three samples follows the same pattern while the growth curve rises similarly with the incubation time (Fig. 3). After 192 hours of incubation, no significant difference between soil samples was observed for carbon substrate utilization (Fig. 4), pledging for the uniformity of the microbial communities regarding their substrate preferences.

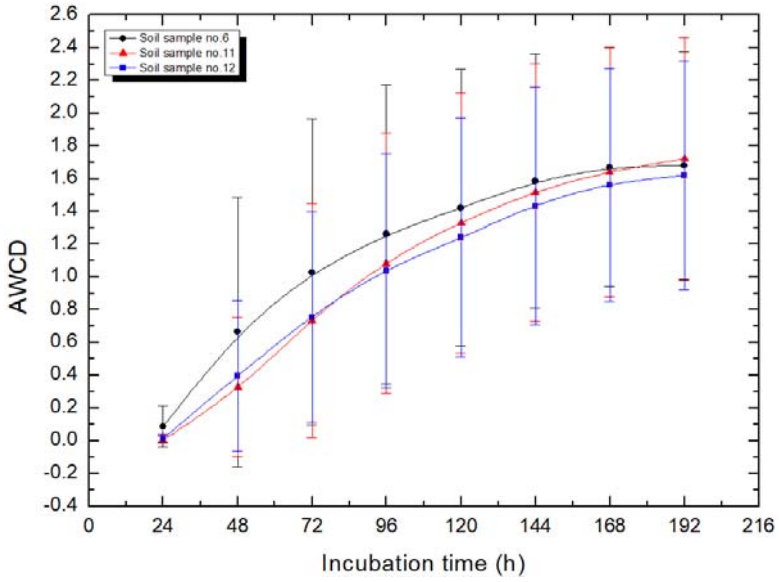

Fig. 3 – AWCD of Biolog Ecoplate carbon substrate utilization.

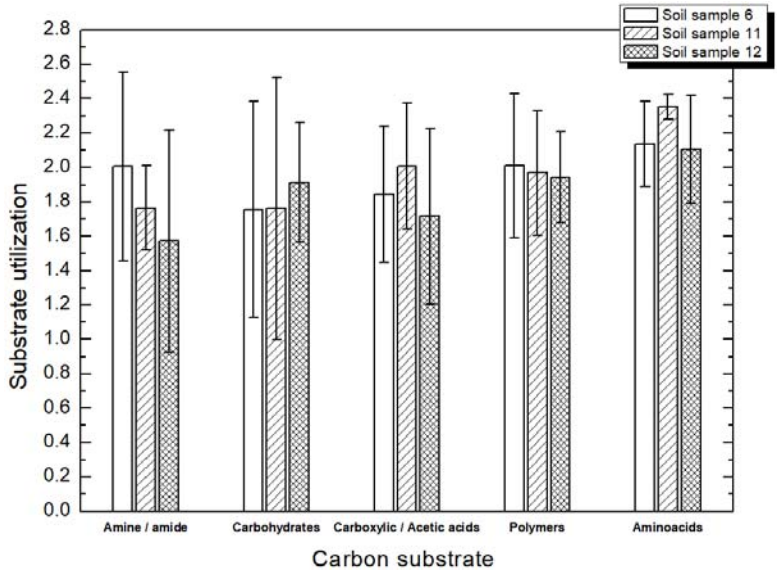

Fig. 4 – Microbial community substrate utilization (OD) after 192 h incubation, grouped by substrate type.

Our isolated microbial communities accepted almost every carbon source present in Biolog Ecoplate, however, no metabolization was observed for some carbohydrates: Glucose-1-phosphate, D-L α glycerol phosphate – soil community

no. 6, no. 11, no. 12, polymer: α-Cyclodextrin – soil community no. 11 and carboxylic and acetic acids: 2-Hydroxy benzoic acid – soil community no. 12.

3.4. THE REDUCTION OF TOXIC ELEMENTS DISSOLVED IN LIQUID CULTURE MEDIUM USING THE OBTAINED RADIORESISTANT MICROORGANISMS

The radioresistant isolates managed to grow in media with high concentration of toxic metals and reduced these concentrations. After a relatively short lag phase, of around 24 h, all microorganisms inoculated in R2 broth supplemented with ^{63}Cu and ^{133}Cs started to grow. The toxicity of ^{59}Co has push the growth lag phase up to around 60 h, with some microorganisms totally inhibited by cobalt presence and the others with timid growth signs.

The toxic effect of ^{59}Co can be also observed in its low reduction efficiency obtained after 7 days of incubation, where only 10 out of 12 microorganisms showed growth (a slight one) and low reduction capabilities (around 3–4%). Only one isolate managed to reach a reduction efficiency of 8%. ^{63}Cu and ^{133}Cs dissolved in the culture medium didn't inhibit the bacterial growth, there was no notable growth difference between control and the tested microorganisms (Fig. 5). Copper reduction capabilities were calculated for every microorganism, the efficiency varying between 10%–19%, while for cesium, the reduction efficiency was a little lower, between 7% and 16%.

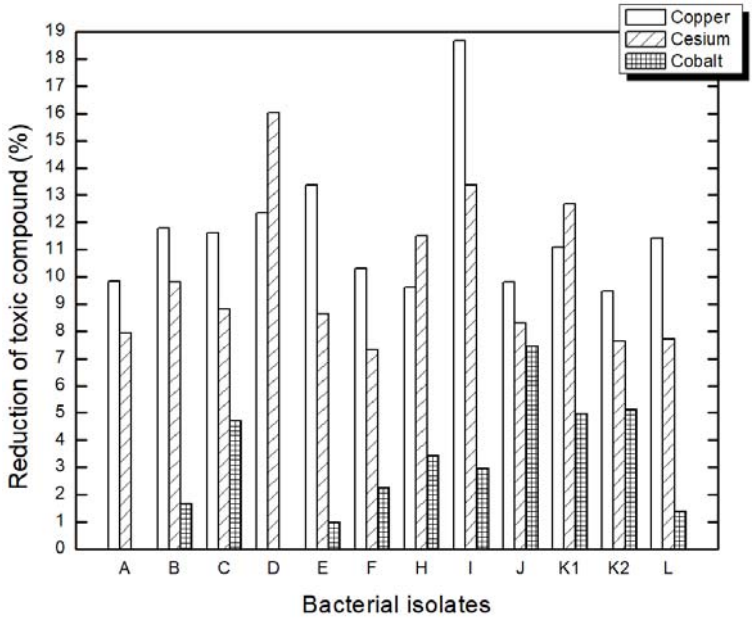

Fig. 5 – Reduction of 1 mM copper, cesium and cobalt added in salt form to culture medium, by the radioresistant isolated bacteria.

4. DISCUSSIONS

These samples, originated from a depleted uranium mine, that at the origin had a high concentration and a high purity degree of uranium ore, contained uranium, potassium, and thorium along with their radioisotopes (Fig. 1).

Naturally, the microbial communities that thrive in this environment will exhibit special characteristics strengthening the the fact that these microorganisms can withstand toxic heavy metals concentrations [16].

Twelve cultivable microbial isolates were obtained from these 3 irradiated soil samples after inoculation on R2 Agar. An extended ICP MS analysis of these soil samples reveals high concentrations of copper, cesium and cobalt (Fig. 6) in all soil samples.

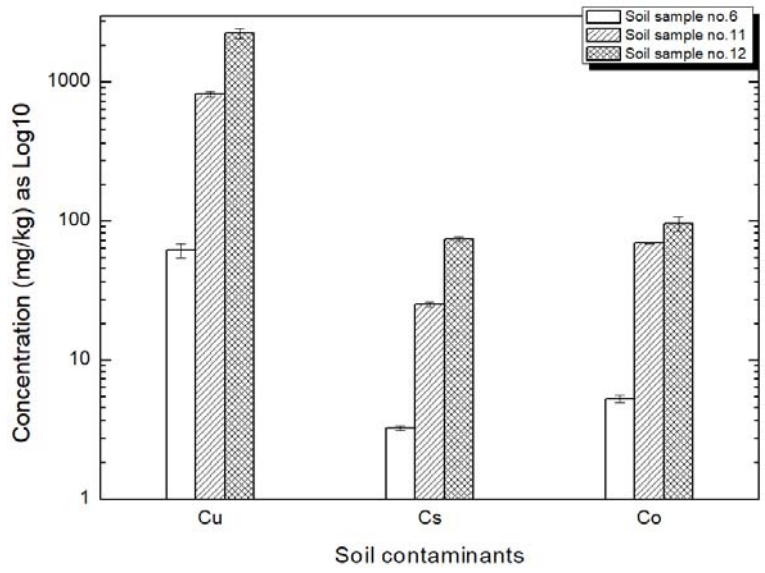

Fig. 6 – Soil contaminants (Cu, Cs, Co) concentration determined using ICP-MS.

Soil sample no. 12 is the most contaminated with heavy metals, displaying the highest concentrations of copper, cesium and cobalt among all three tested samples. Thus, sample no. 12 displayed cooper at 35.27 M, a concentration 36 times higher than sample no. 6 and 2.7 times higher than sample no. 11, cesium at 0.22 M, 2.5 times higher than sample no. 6 and 2.8 time higher than sample no. 11 and cobalt 1.62 M, 18 times higher than sample no. 6 and 1.37 times higher than sample no. 11. Cesium (^{133}Cs) and cobalt (^{59}Co), as non-radioactive elements, are mildly toxic for the environment and human health in low concentrations, while their radioactive isotopes ^{137}Cs and ^{60}Co are byproducts of nuclear fission processes

with half-life of 5.27 years for ^{60}Co and 30 years for ^{137}Cs [17]. These radionuclides pose a great danger to the environment and human health due to their interference in the biochemical cell processes and to DNA damage by irradiation. ^{137}Cs can enter into the bacterial cell and due to its physiochemical resemblance to potassium, it can interfere with K^+ homeostasis. ^{60}Co can also enter inside the bacterial cell where it competes with iron, altering the synthesis of essential metabolic proteins, coenzymes [18], resulting in their inactivation.

Resistant bacteria able to withstand increased concentrations of cesium and cobalt isolated from a nuclear fuel storage pond was reported to exhibit increased tolerance to 500 mM CsCl and 3 mM CoCl. Copper is known to have antibacterial properties, with toxic effects for all living beings due to its ability to produce reactive oxygen species that can damage the DNA and iron-sulfur cluster dehydratase [19] and also a toxic heavy metal inducing a viable but nonculturable condition for *Escherichia coli* [20]. The bacterial ability to remove toxic elements from culture mediums is widespread due to their fluid metabolism and also their fast life cycle [21–23].

Every tested microbial isolate grown in liquid culture medium supplemented with 1 mM toxic metal, displayed the ability to reduce the metal concentration with various efficiency (Fig. 6). The soil bacterial isolates managed to grow and also reduced copper concentration with some efficiency: after 7 days of incubation, the removal efficiency varied between 8% and 19%. A certain metal toxicity pattern can be observed in Fig. 6 from bacterial response to 1 mM concentration (in salt form), with cobalt being the most toxic and copper being the least toxic metal. Isolated bacteria characteristics: all the soil isolated microorganisms are Gram positive, spore forming rods. The identification of these microorganisms was performed using a semiautomated Biolog Identification system (Biolog®). Few strains were identified so far, where A and C being *Bacillus cereus* and D and E being *Bacillus licheniformis*. Both *Bacillus cereus* and *Bacillus licheniformis* are well known as microorganisms capable of bioaccumulation and bioremediation processes, the reduction of cesium and copper being performed by these microorganisms with various efficiency [24, 25].

5. CONCLUSIONS

This paper reveals the ability of microbial communities isolated from a nuclear waste repository soil to withstand high radiation background and high concentrations of toxic heavy metals in the culture medium. We show that selective microbial isolates from a heavy metal and radionuclides contaminated soils, able to withstand gamma radiation exposure at 4 kGy/h dose rate, are also able to remove ^{63}Cu, ^{133}Cs, and ^{59}Co isotopes from the culture medium.

We conclude that radioresistence test can be applied as a tool to select the most efficient microorganism in heavy metal remediation processes, as well as in radionuclides remediation strategies. Microbial communities from restrictive environments display interesting abilities that can be exploited in bioremediation processes. In this study, the bacterial isolates obtained from 3 soil samples with above the normal levels of radioactivity, displayed various abilities. Although the community metabolic fingerprint was almost identical for all three, their radio-resistance was different. These microorganisms proved to withstand increased levels of uranium, potassium, and thorium, as well as to rapidly remove copper, cesium and cobalt from aqueous new environments, while adapting to them.

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