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Community level physiological profiles of bacterial communities inhabiting uranium mining impacted sites

Anelia Kenarova [a,*], Galina Radeva [b], Ivan Traykov [a], Silvena Boteva [a]

[a] Faculty of Biology, Sofia University "St. Kl. Ohridski", 8 Dragan Tsankov Blvd., 1164 Sofia, Bulgaria
[b] Institute of Molecular Biology, Bulgarian Academy of Sciences, Acad. G Bonchev Str., bl. 21, Sofia 1113, Bulgaria

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ABSTRACT

Bacterial activity and physiological diversity were characterized in mining and milling impacted soils collected from three abandoned uranium mine sites, Senokos, Buhovo and Sliven, using bacterial dehydrogenase activity and Biolog (EcoPlate) tests. The elemental composition of soils revealed high levels of uranium and heavy metals (sum of technogenic coefficients of contamination; TCC_{sum}) pollution as follows: Sliven (uranium – 374 mg/kg; TCC_{sum} – 23.40) > Buhovo (uranium – 139.20 mg/kg; TCC_{sum} – 3.93) > Senokos (uranium – 23.01 mg/kg; TCC_{sum} – 0.86). The physiological profiles of the bacterial community level were site specific, and indicated intensive utilization of polyols, carbohydrates and carboxylic acids in low and medium polluted environments, and *i*-erithrytol and 2-hydroxy-benzoic acid in the highly polluted environment of Sliven waste pile. Enzymes which take part in the biodegradation of recalcitrant substances were more resistant to pollution than these from the pathways of the easily degradable carbon sources. The Shannon index indicated that the physiological diversity of bacteria was site specific but not in line with the levels of pollution. A general tendency of increasing the importance of the number of utilizable substrates to bacterial physiological diversity was observed at less polluted sites, whereas in highly polluted sites the evenness of substrate utilization rate was more significant. Dehydrogenase activity was highest in Senokos upper soil layer and positively correlated ($p < 0.01$) with the soil organic matter content. The bacterial activity (EcoPlate) and physiological diversity (Shannon index) correlated significantly and negatively with As, Cu, Zn, Pb and U, and Co, Cr, Ni and Mn, respectively. We concluded that the observed site specific shifts in bacterial communities were complex due to both the environmental peculiarities and the bacterial tolerance to the relevant level of pollution, rather than a strong indication of uranium and heavy metals toxicity.

1. Introduction

Uranium (U) mining and milling activities release into the environment uranium, heavy metals (HMs), and inorganic species, having serious negative effects on wildlife and humans (Okabayashi et al., 2005). The pollutants alter the structure (Gleeson et al., 2006; Kandeler et al., 2000) and metabolic activity (Gong et al., 2002; Kandeler et al., 2000; Kenarova and Radeva, 2010, van Beelen et al., 2004) of soil microbial communities, decrease the microbial number/biomass (Wang et al., 2007), and increase the frequency of uranium and heavy metals resistant bacteria (Davis et al., 2004; Satchanska et al., 2005, van Beelen et al., 2004). The rate and range of heavy metals' toxicity depend on soil type, the level of contamination and the time of exposure (Kozdrój and van Elsas, 2001; Vig et al., 2003). These relationships indicate different adverse effects of pollutants on bacteria depending on local environmental peculiarities which can support bacteria to overcome the stress (Bååth and Anderson, 2003; Nannipieri et al., 2003), and/or modulate the behavior of pollutants including their bioavailability and toxicity (Ramakrishnan et al., 2011). The granulometric composition of soil, quantity and quality of organic matter, pH, total exchange capacity, nutrient availability, moisture, temperature and oxygen availability are among the important soil properties influencing uranium and heavy metals toxicity (Bååth and Anderson, 2003; Boivin et al., 2006). For instance, Kunito et al. (1999) assumed that the nutrient conditions have a greater influence than Cu toxicity on the size of soil microbial biomass even in the highly Cu-

Abbreviations: AWCD, Average well color development; CLPP, Community level physiological profile; Dha, Dehydrogenase activity; HMs, Heavy metals; INT, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride; INT-F, INT- formazan; OM, Organic matter; PCA, Principal component analysis; SAWCD, Substrate average well color development; TCC, Technogenic coefficient of contamination; U, Uranium

* Corresponding author.
E-mail addresses: akenarova@abv.bg (A. Kenarova), gradeva@bio21.bas.bg (G. Radeva), itraykov@biofac.uni-sofia.bg (I. Traykov), silvenab@abv.bg (S. Boteva).

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contaminated soils. In this context, it is reasonable to expect different local effects of uranium and heavy metals on bacteria even depending on season and weather.

Some bacterial parameters can be used as sensitive indicators for the toxicity of uranium and heavy metals, but unfortunately no single method can give complete information about the microbial community which is under stress (Garbeva et al., 2004). Molecular techniques such as 16S rRNA sequences and metagenomic analyses reveal the composition of microbial communities, while other methods like enzyme assays and Biolog test (EcoPlate™) characterize their metabolic activity and physiological diversity. The most sensitive and reproducible is the assay of dehydrogenase activity (Oliveira and Pampulha, 2006; Venkateswar et al., 2010; Vivas et al., 2008), whereas Biolog EcoPlate™ is a fast and commercially available test designed to estimate the community level physiological profile of bacteria (Preston-Mafham et al., 2002). There are; however, several problems in the measurement and interpretation of EcoPlate™ results considering the selectivity of the test to the fast growing aerobic heterotrophic bacteria (Mills and Garlands, 2002; Van Elsas and Rutgers, 2005), and the drawbacks of culturing methods (Garland, 1997). Nevertheless, it provides a rapid and reproducible approach to assess the shifts in metabolite profiles of stressed bacterial communities (Classen et al., 2003; Preston-Mafham et al., 2002).

In Bulgaria, the intensive uranium mining and milling was performed between 1946 and 1991 causing significant soil and water pollution. The uranium production was stopped by Government decree, and since 1992 the uranium mines and tailings were technically liquidated and remediated. However, the mine areas, some of which are agricultural lands, continue to be territories with high pollution by radionuclides and heavy metals.

The aim of this study was to determine the impact of uranium and heavy metals pollution on metabolic activity and physiological diversity of bacteria, using the dehydrogenase activity assay and the Biolog EcoPlate™ technique. We hypothesized that complex uranium and heavy metals pollution results in long-lasting impact on bacteria reducing their metabolic activity and physiological diversity, and these shifts in bacterial communities are site specific. If our hypothesis is correct, we expect to differentiate clearly the site specificity of bacterial carbon utilization rate and pattern, corresponding to both the local level of pollution and the environmental characteristics.

2. Materials and methods

2.1. Sites and sampling

Three sites in Bulgaria impacted by uranium mining activities were studied – the mining and milling complex Buhovo, the Senokos mine and the mine at Sliven. The mining complex "Buhovo" (42°45′51.20″N; 23°34′36.86″E) is located 30 km northeast of Sofia on a territory of 2280 ha, the mine Senokos (41°49′53.00″N; 23°13′11.80″E) is in the southwestern part of the country on a territory of 17.92 ha, and the mine at Sliven (42°41′47.68″N; 26°22′22.47″E) mine is in southeastern Bulgaria, occupying an area of 491 ha (Fig. 1). Mining operations in Buhovo and Sliven had been conducted in a conventional underground manner while Senokos was an open-cast mine. The mines were in operation till 1991, completely closed in 1992, and remediated until 2001. The sampling areas were secondary grass lands dominated by *Poaceae* (Senokos, Buhovo, Sliven), *Caryophyllaceae* (Senokos, Sliven), *Fabaceae* (Senokos, Buhovo), *Asteraceae* (Sliven), *Apiaceae* (Buhovo), and *Brassicaceae* (Senokos).

Three samples (0.5 kg for chemical- and 50 g for microbiological analyses) from each site (12 m^2) were collected in May 2009, 2010 and 2011 from the upper (0–5 cm) (SU 1p, SU 5 and SU 22 and BuhC) and the deeper (35–40 cm) (SU 1d and BuhD) soil layers of Senokos (SU) and Buhovo (Buh) mine areas. The sample named "Sliv" was collected also in May 2009, 2010 and 2011 from the Sliven mine waste pile at a depth of 40 cm. Samples for microbiological analysis were collected by soil probe under sterile conditions, transported at 4 °C, and the analysis was done within 3 days. Samples for chemical analysis were transported in plastic containers, dried at room temperature, sieved (2 mm) and analyzed.

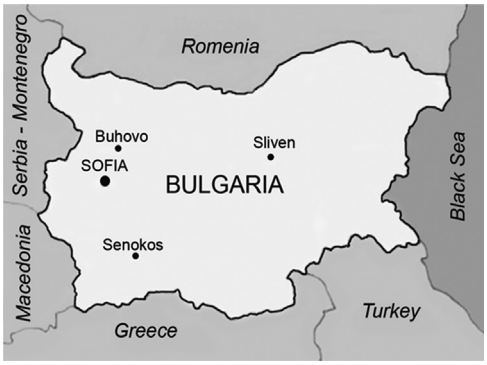

Fig. 1. Location of the areas impacted by uranium mining activities: mine Senokos (sampling sites SU 1p, SU 1d, SU 5 and SU 22), mine Buhovo (sampling sites BuhC and BuhD), and mine Sliven (sampling site Sliv).

2.2. Environmental variables

The soil organic matter (OM) content was determined by Turyn's method based on its oxidation by potassium dichromate (Kaurichev, 1980). The pH_{H2O} was measured potenciometrically (HANNA pH-meter). The concentrations of sulfates, inorganic nitrogen (nitrates and ammonia), and phosphates were determined spectrophotometrically by the methods of Bertolacini and Barney (1957), Keeney and Nelson (1982), and Olsen (1982), respectively.

The concentrations of heavy metals, uranium, and the metalloid arsenic (As), were analyzed by an ELAN 5000 Inductively Coupled Plasma Mass Spectrometer (Perkin-Elmer, Shelton, CT, USA) in 1 M HCl solution (1:20; soil:1 M HCl) according to ISO110 47:1998 (heavy metals) and Bulgarian state standards (As and U). The analyses were carried out by the accredited laboratories of DIAL LTD (⟨http://dial-ltd.com⟩), specialized in analyses of radionuclide and heavy metals. All concentrations were calculated for oven-dried soil.

2.3. Microbial community analyses

Bacterial dehydrogenase activity (Dha) was assayed under standard conditions by the method based on reduction of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) to INT-formazan (INT-F) according to Tabatabai (1994). Briefly, samples were placed into test tubes and 2.5 ml of INT-Tris buffer (0.1 M Tris–HCl, pH 7.0, 0.3 percent INT) were added. The tubes were incubated at 25 °C for 1 h (SU, Buh, Sliv) and 24 h (Sliv). The reaction was stopped by adding 0.2 ml ice acetic acid. INT-F was extracted three times with 10 ml of methanol for 30 min in the dark. The absorbance was measured at 485 nm and the amount of INT-F (μg F/g/h) was calculated according to a calibration curve.

Average well color development (*AWCD*) and community level physiological profile (CLPP) were analyzed using Biolog EcoPlates™, containing 31 different carbon (C) sources in three replicates (Biolog Inc., Hayward CA, USA). The plates' wells were inoculated with 120 μl bacterial cell suspensions (1 g soil in 50 ml sterile 0.9 percent NaCl, suspended for 30 min at 240 × g followed by filtration through 8.0 μm and 3.0 μm pore size membranes) and the plates were incubated at 25 °C in the dark for 7 days. Substrate utilization was monitored by measuring absorbance at 590 nm using Microplate Reader LKB 5060-006 and software package DV990 "Win 6". The measurements of individual substrates were corrected for background absorbance by subtracting the absorbance of control (water) sample. The color development of each plate was expressed as *AWCD* suggested by Garland and Milles (1991):

$$AWCD = \sum \frac{(n_i - c)}{31},$$

where n_i and c were the optical densities (*ODs*) of the substrate (where $i = 1$–31) and control wells, respectively.

The Ecoplate substrata were grouped into five biochemical categories following Sala et al. (2010), but the originally grouped carbon sources as carbohydrates (*i*-erythritol and D-mannitol) and polymers (tween 40, tween 80, cyclodextrin and glycogen) were rearranged into categories of polyols (*i*-erythritol, D-mannitol, tween 40, and tween 80) and carbohydrates (cyclodextrin and glycogen). The color development of biochemical categories was expressed as substrate average well color development (*SAWCD*).

2.4. Statistical analysis

The analyses were performed in triplicates for each sample, and the results in the study were presented as mean values for the three years of sampling (± standard deviation) avoiding the inter-annual variation. One-way ANOVA and the Tukey-Kramer HSD test ($p < 0.05$) were used to evaluate the differences