Bioremedijacija ugljenom onečišćenog tla pomoću bakterijskih kultura (Istarski ugljenokopi Raša)

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Decontamination of soil polluted with coal using bacterial biomass (Istrian Raša coal mines)

Bioremedijacija tla onečišćenog ugljenom (Istarski ugljenokopi Raša)

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This Master thesis was made at the Department of Geology at the Faculty of Science under the guidance of Associate Professor Gordana Medunić at the Mineralogical-petrographic Division of the Geological Department of the Faculty of Science and in Bioremediation Lab of the Department of Botany of the Banaras Hindu University under the guidance of Assistant Professor Asha Lata Singh as part of the Master in Environmental Sciences at the Faculty of Science, University of Zagreb.

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BIOREMEDIJACIJA UGLJENOM ONEČIŠĆENOG TLA POMOĆU BAKTERIJKIH KULTURA (ISTARSKI UGLJENOKOPI RAŠA)

Magdalena Janeš

Rad je izrađen na Mineraloško-petrografskom zavodu Prirodoslovno-matematičkog fakulteta, Sveučilište u Zagrebu, Horvatovac 95, 10 000 Zagreb, Republika Hrvatska te u Bioremedijacijskom laboratoriju na Botaničkom odsjeku, Hindu sveučilišta u Banaresu, 221005 Varanasi, India.

Sažetak: Tlo prikupljeno s područja Labinštine onečišćeno je Raškim ugljenom i pepelom kao rezultat višedesetljetnih rudarskih i metalurških aktivnosti te rada TE Plomin. Dosadašnji objavljeni radovi ukazuju na povišene vrijednosti sumpora te niza potencijalno toksičnih elemenata u tragovima. Stoga je korištena bioremedijacijska metoda jer se smanjenjem potencijalne toksičnosti tla i umanjenjem negativnog utjecaja na osjetljiv krški i marinski obalni ekosustav, poboljšava kvaliteta okoliša. U ovom je radu ispitano smanjenje sumpora. Eksperiment se temelji na korištenju bakterijske kulture *Ralstonia sp.* koja svojim metabolizmom uklanja sumpor iz ispitivanog materijala. Rezultati su pokazali relativno veliko smanjenje u dva uzorka tla sa smanjenjem sumpora od 69,75 % u uzorku Plomin te 47,83 % u uzorku Štalije dok je uzorak ugljena pokazao relativno malo smanjenje sumpora od 5,64 %.

Ključne riječi: desulfurizacija, *Ralstonia sp.*, Raški ugljen, SHOS ugljen, tlo

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BIOREMEDIATION OF COAL CONTAMINATED SOIL WITH BACTERIAL CULTURES (ISTRIAN COALMINES RAŠA)

Magdalena Janeš

Thesis completed at the Division of Mineralogy and Petrology, Faculty of Science, University of Zagreb, Horvatovac 95, 10 000 Zagreb, Croatia and in Bioremediation Lab, Department of Botany, Banaras Hindu University, 221005 Varanasi, India.

Abstract: Soil collected from the Labin city area is contaminated with Raša coal and ash because of several decades of mining, metallurgical activities and coal combustion in a Plomin thermal power plant. Recent publications have shown elevated values of sulfur and several potentially toxic trace elements in that soil. Therefore, to improve the quality of the environment, bioremediation method was used to reduce potential soil toxicity and negative impact on the sensitive karst and marine coastal ecosystem. In this thesis removal of sulfur (in %) was examined. The experiment is based on use of bacterial cultures of *Ralstonia sp.* which, owing to its metabolism, remove sulfur from the investigated material. Results are showing relatively high removal in two soil samples with sulfur removal of 69,75 % in the sample Plomin, and removal of 47,83 % in the sample Štalije while coal sample showed relatively low sulfur removal of 5,64 %.

Key words: desulfurization, *Ralstonia sp.,* Raša coal, soil, superhigh-organic-sulfur (SHOS) coal

Thesis contains: 48 pages, 16 figures, 1 table, 61 references

Original in: English

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CONTENT

1. INTRODUCTION

Coal is an organogenic, sedimentary rock. Although it originates mainly from plant residues, there are some types of coal where animal residues can be found in its composition. Coal was used for thousands of years but not until appearance of steam engine and industrial revolution, it became greatly popularized which had as a result increased impact of coal burning on the environment. Nowadays coal is the most common energy source. It plays an important role in meeting global energy needs and is critical to infrastructure development because 37 % of the world's electricity and 74 % of the world's steel is produced using coal. Increasing development of technology and industry brings its negative sides. From ecological aspect, coal is one of the largest producers of pollution in field of energy sources (World Coal Association, n. d.).

1.1. Coal

Coal is a fossil fuel originating mainly from vegetation accumulated in swamps and peat bogs millions of years ago. When we start from the beginning, coal wouldn’t exist if there is no process called photosynthesis. All living plants collect energy from the sun in this process. After plant death and decay, energy stored through years in plant cells start releasing. Certain environmental conditions disable this process, so that energy is preserved in form of coal. Formation of coal began in Carboniferous which lasted for 70 million years, from 360 to 290 million years ago. Plant material accumulated in swamps and peat bogs ended often on great depths due to tectonic movements and sedimentation of younger sediments. Because of that, most coals occur in stratified sedimentary deposits if there were no intrusions of igneous rocks or deformations. With depth, temperature and pressure also increase. Under that conditions, a lot of chemical and physical changes happen to primary plant material which alternates into peat and then into coal (World Coal Association, n. d.). Almost all elements of periodic table can be found in the composition of coal. They can be divided in three groups: macroelements (C, H, O, N, S) with concentration above 1000 mg / kg; microelements (Si, Al, Ca, Mg, K, Na, Fe, Mn, Ti, F, Cl, Br, I) with concentration between 100 and 1000 mg / kg; trace elements (Cu, Zn, Pb, Cr, Ni, Hg, Se, As) with concentration lower than 100 mg / kg (Rađenović et al., 2016).

Coalification is a process of transforming plant material into the coal. There are different degrees of coalification resulting with different types of coal (from peat to anthracite). The difference between coal ranks or degree of coal metamorphism is in carbon content and volatile matter. The ranks of coal going from the one with the least carbon are lignite, subbituminous, bituminous and anthracite. Variety of coal will arise because of differences in plant material, depths of burial, environmental conditions (temperature and pressure) and duration the coal has been forming (Encyclopaedia Britannica, n. d.).

Two methods of coal mining are present, surface mining or opencast and underground mining. Type of geology of the coal deposit is the main criteria for the choice of mining. Underground mining makes bigger share of world coal production. 6.9 billion tons of hard coal are currently produced worldwide. Coal plays an important role in electricity generation, in transport and in industry. For example, steel production is dependent of coal because 70 % of it is made with the energy generated from coal (World Coal Association, n. d.).

1.1.1. Coal structure

There are three main parts of coal: organic matter, ash and water. Organic matter is consisting primarily of carbon, hydrogen, oxygen, sulfur and nitrogen. Ash is lagging after coal combustion and is composed of inorganic compounds such as carbonates, oxides, sulfates, sulfides, silicates, clay, salts that are introduces into the coal from the parent organic matter (Hrvatska enciklopedija, n. d.).

Maceral is organic constituent of coal with characteristic physical and chemical properties. They don’t have crystalline structure although are analogous to minerals in inorganic rocks. They change in accordance with coal rank. They are classified into three groups: vitrinite, inertinite and liptinite. Vitrinite is derived from cell walls and woody plant material and most coals contain high percentage of it (50 to 90 %). Inertinite is a group of macerals rich in carbon and most coal contain 5 to 40 % of it. They are formed from plant material which went through a severe degradation during the peat stage. Liptinite is the group of macerals with high hydrogen content which are derived from the cuticles and resin. It is the least present in coals with 5 to 15 % (Kopp, 2018).

1.2. Coal-caused pollution

Coal mines and coal processing have affected environment negatively. Coal mining demands pumping out large quantities of groundwater to access the coal. Mine slurry seeps into local water supplies. The water is also used for cooling the industrial structure and to remove pollutants such as nitrogen oxides, sulfur (IV) oxides and mercury causing further pollution (Greenpeace, n. d.). The incomplete burning of coal produces many compounds, some of which are carcinogenic (Figure 1). Sulfur and nitrogen oxides that are produced react with atmospheric moisture producing sulfuric and nitric acid (acid rain) (Encyclopaedia Britannica, n. d.). Coal can be enriched with heavy metals such as Cd, As, Ti, Mn, Zn, Pb, Fe, Rb, Sr, Nb, Zr and others (Bhuiyan et al., 2010). The burning of coal also releases particulate matter like fly ash that can be transported by wind for long distances, and solids such as bottom ash and slag that are deposited at the source site (Encyclopaedia Britannica, n. d.). That particles contain these heavy metals that can be transported with ash and deteriorate quality of soil and water. Also, a large quantities of carbon (IV) oxide are released with burning of coal in the atmosphere preventing longer-wavelength rays radiated from the Earth’s surface to escape into space. In the process of mining huge amounts of water are discharged to surrounding area to facilitate the mining. Such water can contaminate surface and ground water (Tiwary, 2001). The burning of coal has negative effect on living organisms. The impact on human health is huge, from possible respiratory illnesses, lung cancer, weakening of the immune system, reduction of lung function, arsenic poisoning, etc. (Zhang and Smith, 2007). Negative impact was also visible on marine and aquatic organisms both directly or indirectly through food web (Siboni et al., 2004). There are several practices that are decreasing undesirable effect of coal mining and processing. The coal management can go in direction of using alternative methods of coal burning (low nitrogen dioxide burners or fluidized bed combustion), or in direction of minimization or removal of dissolved and suspended pollutants in the waste discharge (Encyclopaedia Britannica, n. d.; Tiwary, 2001).

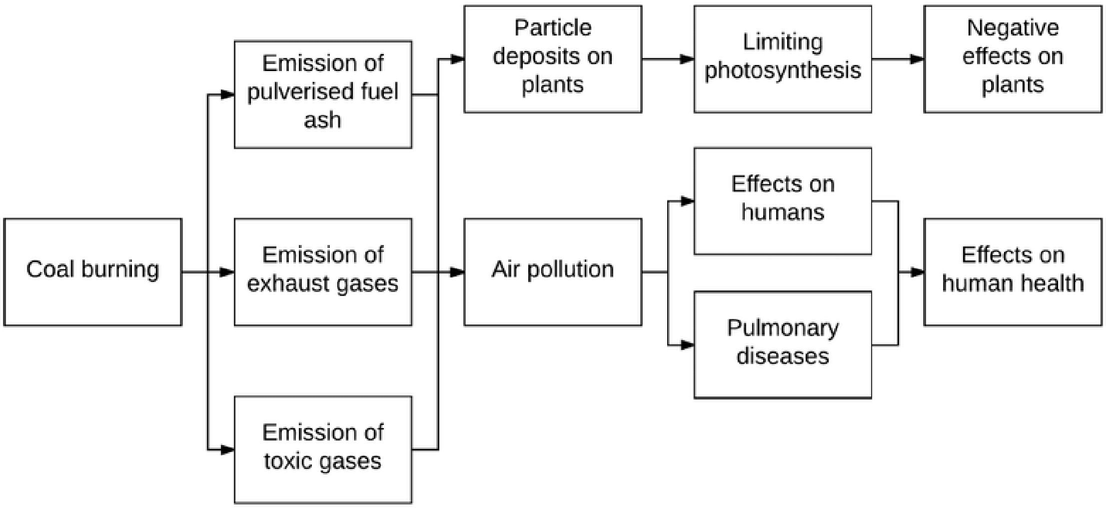

Figure 1. Environmental impacts of coal burning. (https://www.researchgate.net/figure/The-environmental-impact-generated-by-coal-burning_fig5_312554307)

1.3. Sulfur

Sulfur is a chemical element that has symbol S in the periodic table of the elements, its atomic number is 16 and atomic mass is 32,065. Sulfur is at ambient temperature light yellow-coloured fine powder without smell. Insoluble in water but soluble in nonpolar solvents such as tetrachloride or toluene. At elevated temperatures, its reactivity increases forming chemical bonds with chlorine, carbon, iron, etc. It has more than 30 allotropic modifications, but the most prevalent allotrope is the elemental sulfur which has an annular structure of eight atoms (cyclo-S_8) connected by single covalent bonds. At room temperature it's polymorph α-sulfur (rhombic structure). The structure is changed in monoclinic (β-sulfur) above 95 °C. In sulfides, sulfur has oxidation number -II. At the formation of other sulfurous compounds, d-orbital of sulfur atom may be involved in the chemical bond causing positive oxidation number of sulfur atom (II, IV, VI) (Filipović and Lipanović, 1985).

It is essential to all living organisms. It's taken up in the form of sulfates from the soil or water by plants and algae. It is compound of two essential amino acids (methionine, cysteine), some co-enzymes crucial in metabolic pathways and part of the strongest antioxidant in our body, glutathione. The average human body contains around 140 g of sulfur (Stanković and Kiralj, 2018). Sulfur and sulfate are nontoxic forms but carbon (IV) sulfide, hydrogen sulfide and sulfur (IV) oxide are toxic. Sulfur and its forms are used in vulcanization of black rubber, in production of sulfuric acid, pesticides and herbicides, for paper bleaching, as food preservatives, in detergents and surfactants, etc. (Filipović and Lipanović, 1985).

Sulfur in coal can be organic or inorganic. The inorganic forms are usually sulfides, sulfates and pyrite. The organic sulfur structures originate from parent material in form of macromolecular structures. It is observed that organic sulfide tends to increase with decreasing rank (Huffman et al., 1991). High-sulfur coals obtain their sulfur mainly from reduction of sulfate ions to H_2S in salty water by bacteria (Calkins, 1994).

SO_4^{2-} + organic matter $\rightarrow S^{2-} + H_20 + CO_2$

$S^{2-} + 2H^+ \rightarrow H_2S$

Although elemental and sulfide sulfur increase the heat value of coal, they are not desirable because the product of their combustion is sulfur (IV) oxide which is known as air pollutant. Also, high sulfur level increases the self-inflammability of coal making it undesirable for storage (Hrvatska enciklopedija, n. d.).

1.4. Bioremediation

Bioremediation is a process of removing or neutralizing contamination into less or nontoxic substances using naturally occurring organisms. It uses no chemicals which makes this type of treatment much healthier for ecosystem. In its essence, bioremediation is intensive enhancement of process of self-cleaning that is naturally occurring in nature (Đokić et al., 2012). In past years, at the beginning of soil management, many chemicals were used for cleaning which showed later to have negative influence for entire well-being of nature (Nyer, 1992). Physical interactions between soil, pollutants and organisms are so diverse which lead to many obstacles in bioremediation. Biodegradation of pollutants in soil is complex process due to diffusion of contaminants, adsorption to the surface of soil particles, degradation in biofilms, pores and water either bound or free (Margesin and Schinner, 2005). Advantages of bioremediation over physical or chemical treatments are lower cost and often no or very little residual treatment is required due to degradation of contaminants. Disadvantages are difficulties in controlling degradation parameter and in degradation of contaminants, longer duration of the process compared to physical or chemical and sensibility of the process itself (Dong, 2017).

The bioremediation itself can be made in-situ or ex-situ. In-situ treatment is made in original locality of contamination which prevents the spread of contaminated substances during the transport (biostimulation, bioaugmentation and bioventing). Ex-situ bioremediation refers to the process where polluted unit is replaced on a different area which helps to control the treatment and its products (bioreactors, composting, biofilters and land farming) (Megharaj et al., 2014). Special types of remediation are phytoremediation and rhizofiltration.

There are few types of bioremediation often used in common practice:

1. Phytoremediation is using plants to clean the contaminated matter from pollutants by assimilating the same and removing from environment.
2. Rhizofiltration is used specifically to remove metals from water using roots of plants.
3. Biostimulation is using microbes specifically designed for removing certain contamination.
4. Bioaugmentation is introducing a large number of exogenous microorganisms into the biotreated system depending of the type of contamination.
5. Bioventing is used for oxygen sensitive metals or chemicals by blowing air through soil which increases oxygen rate.
6. Bioreactor is using containers to maintain the contaminated soil in slurry suspension while bioremediation occurs under controlled conditions.
7. Composting means natural decay and remediation under controlled conditions by microorganisms.
8. Biofilters is method of capturing and sequestering pollutants long enough to allow biology to work. Contaminants are exposed to aerobic microorganisms once trapped in a filter media containing compost.
9. Land farming is technology where contaminated soil is placed onto lined beds and aerated by periodically turning over or tilled. Soil conditions such as moisture content,

aeration, pH, nutrients, etc. are controlled for optimum rate of contaminant degradation (Megharaj et al., 2014).

1.4.1. Bacterial remediation

Bacterial remediation is one of the methods used for restoration of soils. The objective is the same, removing contaminants and pollutants. Bacteria are normally found in soils contributing to latter's improvement. Either aerobic or anaerobic or facultative anaerobic, bacteria are helping with nitrogen fixation, decomposition of heavy metals, pesticides and polycyclic aromatic hydrocarbons (PAHs) and limitation of growth of plant pathogens in the soil (Crawford and Crawford, 1996). The bacteria's utilization of contaminants and pollutants must be useful to them otherwise remediation process wouldn't occur. They are implementing certain elements and closing their life cycle. Also, bacteria are provided with carbon and energy from hydrocarbons (Crawford and Crawford, 1996).

A main objective of bacterial remediation design is to remove the limiting factors in their growth (Nyer, 1992). Sometimes environmental conditions are not appropriate for proper work of bacteria. For improvement of cleaning treatment, pure bacteria cultures are isolated from environment itself. Bioremediation process may be rendered by naturally occurring bacteria or introduced inoculum. If coal or soil carries bacteria capable of desulfurization, then the use of these organisms seems to be a good way of purifying. The use of such organisms can reduce expenses of the hole process and avoid the possible problems in the adaptation of introduced organisms (Aller et al., 2001). Required nutrients (nitrogen, phosphorus, trace minerals), quantity of moisture, ideal pH, introduced oxygen or other electron acceptors and adequate temperature can be provided to optimize a natural process depending of what is needed. Also, variables that may be controlled are biodegradation of pollutants and their adsorption to soil particles (Crawford and Crawford, 1996). In other cases, bioaugmentation is used to clean treated system when naturally occurring bacteria are not effective or sufficient (Margesin and Schinner, 2005). Genetically engineered bacteria (GEM) are playing an important role in soil restoration. Unnaturally occurring pollutants cannot be totally degraded by bacteria. Therefore, GEM that are evolved for specific pollutant or contaminant either naturally occurring or made by human, are becoming a part of bioremediation process (Singh et al., 2011).

Bioremediation processes are basically oxidation-reduction reactions also called redox reactions. Some chemicals are donators of electron (electron donor) being oxidized in the same time while others are receiving the electron (electron acceptor) and being simultaneously reduced (Filipović and Lipanović, 1985). Common electron acceptors in bioremediation processes are oxygen, nitrate, sulfate, carbon (IV) oxide, oxidized metals like iron (III), manganese (III, IV). Electron donors include variety of organic compounds like sugar, fats, fuel hydrocarbons and reduced organic pollutants (Crawford and Crawford, 1996). Chemolithotrophic bacteria use energy obtained from the oxidation of inorganic compounds. Sulfur oxidizing bacteria convert elemental or inorganic sulfur into sulfate:

$2\ S + 3\ O_2 + 2\ H_2O \rightarrow 2\ H_2SO_4$ (*Thiobacillus thiooxidans*)

$12\ FeSO_4 + 3\ O_2 + 6\ H_2O \rightarrow 4\ Fe_2\ (SO_4)_3 + 4\ Fe\ (OH)_3$ (*Thiobacillus ferrooxidans*) (Singh, 2010).

1.5. Bacteria

Bacteria are prokaryotic, microscopic and single-celled organisms living in almost every part of Earth. They have double-stranded DNA that is not enclosed with nuclear membrane. The average bacterial cell diameter is 1 µm. They can use almost every organic compound and some inorganic as a food source. The different bacterial shapes are bacillus (rod), coccus (spherical), spirillum (spiral), spirochete (corkscrew), vibrios (comma), chain of cocci, cluster of cocci, pair of cocci and chain of bacilli (Encyclopaedia Britannica).

Bacterial cell is different in many features from eukaryotic (Figure 2). It doesn’t have organelles (membrane-enclosed structures separated from cytoplasm) that are typical for eukaryotic. It has equivalent of nucleus called *nucleoid*. It’s bacterial chromosome, a carrier of genetic information. Like mentioned before, it consists of circular double-stranded DNA. *Capsule* is outer layer of cell mainly made from polysaccharides or polypeptides. It’s tightly attached to bacterium and has definite boundaries. The role of capsule is to protect cell from drying, antibiotics, chemical agents, phagocyte, and also serves to attach cell on firm surfaces and aggregate bacteria. Many bacteria are motile. Extracellular appendages they possess called *flagella* are allowing them swimming or gliding. Flagellum is long filament and depending on their position on the cell, exists four types of bacteria: monotrichous (with a single flagellum), lophotrichous (with many flagella on one end), amphitrichous (with single flagellum on both opposite ends) and peritrichous (with many flagella across entire surface). Simultaneous movement of flagella is important for moving towards the source of food and for distancing from negative influences. Other type of extracellular appendages are *pili* with which bacteria can adhere to a surface. They are present only in gram-negative bacteria. Sexual pilum or F-pilum is different from the others and play an important role in mating process called conjugation. *Cell wall* is layer surrounding protoplast and is supporting bacterial cell ensuring permanent shape. It protects cell from negative changes of osmotic pressure and mechanical damages. According to cell wall material, defer Gram-positive and Gram-negative bacteria which is one of basic features in their determination. The cell wall of Gram-positive bacteria contains around 95 % of murein. A murein (lat. *murus* - wall) is a peptidoglycan only found at prokaryotes. It is more less porous which allows entry of substances. That's the reason why Gram-positive bacteria are easy to paint with crystal violet but hard to wash with ethanol or acetone due to thicker layer of murein that acts like a barrier. Under the microscope, they will be purple. Also, the cell wall of Gram-positive bacteria contains teichoic acid. Gram-negative bacteria have cell wall of 5-12 % of murein, without teichoic acid but with larger content of lipopolysaccharides, lipopolyproteins and phospholipids. Under the microscope, they are recognized as pink cells after washing with ethanol and counterstaining with safranin. Bacteria without the cell wall are called mycoplasmas. *Protoplast* of bacterial cell is surrounded with cell membrane. In protoplast all processes needed for functioning of the cell are carried out. Functions like cell breathing and production of energy. In the protoplast numerous ribosomes are spread on which the synthesis of proteins takes place. *Cell membrane* is composed of proteins and lipids. It prevents the loss of cell's content, regulates the flow of nutrients and is secreting extracellular enzymes in ambience allowing disintegration of large molecules of food and entry of smaller particles into the cell. The cell membrane has invaginations which increase the production of energy (Duraković and Redžepović, 2002).

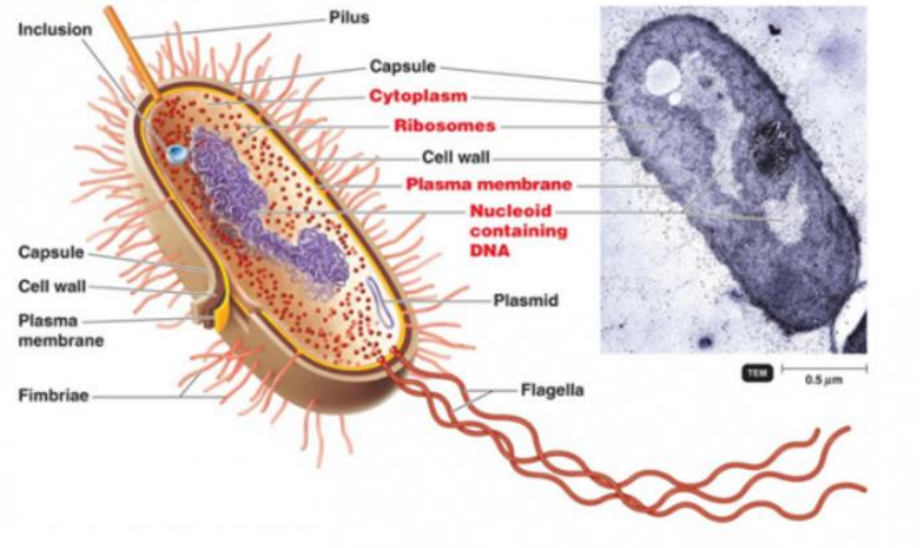

Figure 2. Schematic representation of the bacterial cell.

(https://glycopedia.eu/Main-Features-of-the-Bacterial-Cell-Wall)

A reproduction of bacteria can be a binary fission or sexual. The binary fission is often way of reproduction where the mother cell grows in volume, divides in half and yield two daughter cells with identical genetic material. A sexual reproduction includes conjugation, transduction and transformation. The conjugation is the transfer of DNA between two bacteria mediated by plasmids. Plasmids are nonchromosomal DNA molecules which can provide new useful characteristic for bacteria such as synthesis of new antigens or resistance to antibiotics. In conjugation participates F-pilum transferring plasmids from donor cell to recipient through conjugal bridge. The transduction is the transfer of DNA from one bacterium to another by bacteriophage (virus infecting bacterium). The transformation means embedding of foreign fragment of DNA into their own DNA. To make it possible, the cell must be competent meaning capable of binding a foreign DNA which naturally occurs in few genders. But it is possible under laboratory conditions to render cells competent with use of different solutions e.g. calcium chloride. The transformation is used in recombinant DNA technologies (Duraković and Redžepović, 2002).

According to way of production of energy and use of carbon, bacteria can be divided in autotrophs (photoautotrophs and chemoautotrophs) and heterotrophs (saprophytes, parasites and symbionts). The biggest number of bacteria belongs to chemotrophs where energy is produced from oxidation of organic or inorganic compounds. The energy is obtained from the oxidation of nitrites, methane, carbon (II) oxide, hydrogen, iron, ammonia, sulfur, etc. (Duraković and Redžepović, 2002).

1.5.1. Phases of bacterial growth

Cell concentration is the number of individual cells per unit volume of a culture and bacterial density is the dry weight of cells per unit volume of a culture. In the growth of a bacterial culture, a succession of phases is characterized by variations of the growth rate. Exist four phases of bacterial growth: lag phase, exponential phase, stationary phase and phase of decline (Figure 3). Each of them will be separately explained.

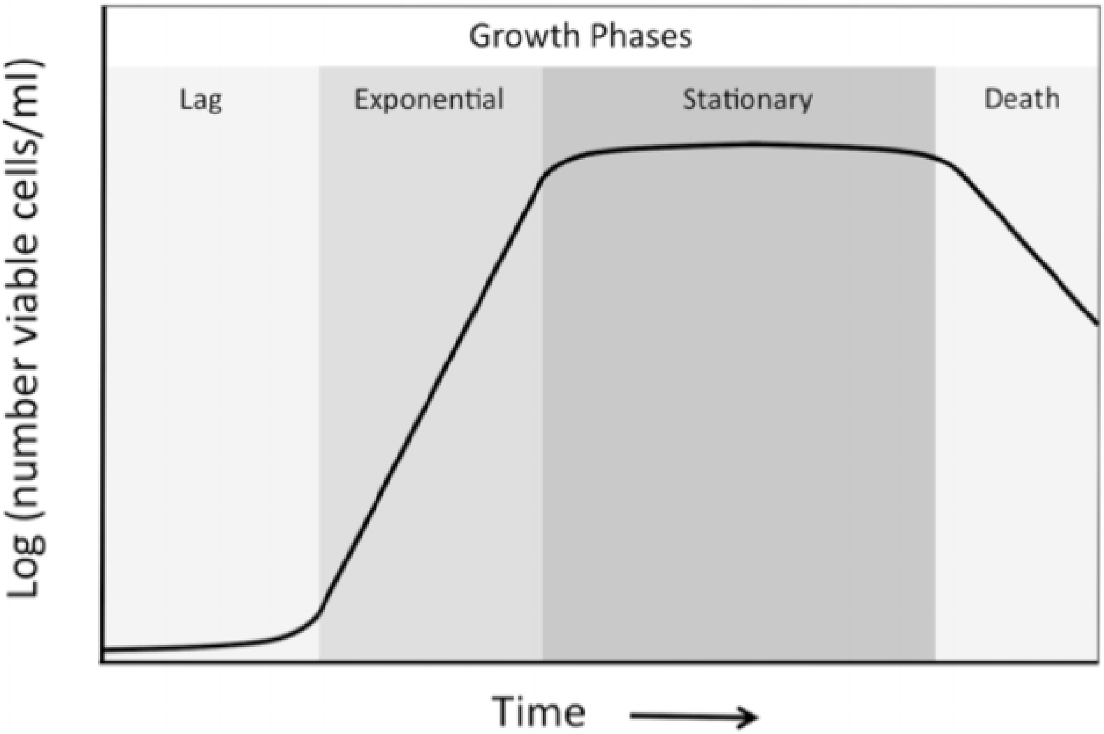

Figure 3. Four phases of bacterial growth.

(https://www.researchgate.net/figure/Growth-of-a-bacterial-population-typically-shows-four-phases-Note-that-the-y-axis-uses-a_fig2_273457358)

1) Lag phase

During the lag phase, bacterial cells are adapting themselves to new conditions. It’s the phase where no cell division is yet present and energy is invested in synthesis of different molecules. There is no increase in cell numbers but, due to cell preparation for reproduction, there may be increase in cell volume. This phase can last for one hour up to several days. The duration of this phase is depending on the age of the bacterial culture. Also, if bacteria were transferred to the different medium, some time will be needed to produce new types of enzymes due to new type of nutrients. This is called enzymatic adaptation.

2) Exponential phase

During the exponential phase, the growth rate is constant, and the steady state is established. The rate of growth represents the velocity of reactions in which cell structure are synthesized. Most of these reactions are reversible enzymatic so the steady state is present when the concentration of all reactants (metabolites) and all catalysts (enzymes) are constant. This phase is represented with a straight line. The slope of this line is the specific growth rate. Bacteria are most active, and the metabolic rate is the highest, because of that bacterial cells from this phase have the biggest activity.

3) Stationary phase

The stationary phase is described with little angle of the growth curve making it horizontal. Bacterial culture is depleting some of nutrients. The accumulation of toxic metabolic products is inhibiting growth as well. All that is resulting in the same rate of the number of new-born cells and the number of dead cells.

4) Phase of decline

During this phase, the number of dying cells is exceeding the number of new-born ones. It is also exponential but, it’s inverse. The phase is ended when the bacterial culture is evanished (Monod, 1949).

1.5.2. *Ralstonia sp.*

Genus *Ralstonia* is relatively new genus that was known before as *Pseudomonas*. All started with *Pseudomonas pickettii* when was transferred to the new genus, along with several other species (Wisplinghoff, 2017).

Ralstonia sp. are Gram-negative, aerobic, rod-shaped bacteria (Figure 4). They can be found both in water and soil living in wide variety of environmental conditions but thrive the best in moist ones (Waugh et al., 2010). It is commonly found in metal rich habitat (Singh et al., 2012). It is shown that *Ralstonia sp.* have capability to degrade toluene, benzene and other organic compounds as well as some heavy metals such as uranium, nickel, cobalt and cadmium (Vroblesky et al., 1997; Utturkar et al., 2013).

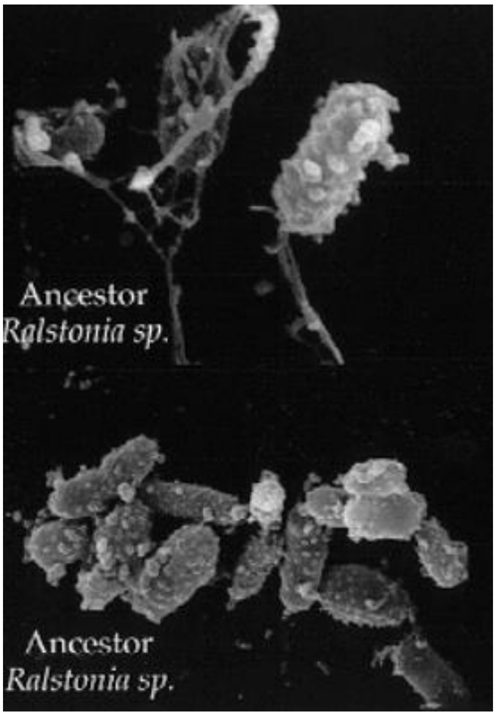

Figure 4. *Ralstonia sp*.

(http://cme.msu.edu/RES_PROG/ralstonia.html)

1.6. Aim of the thesis

The aim of this work is to learn a soil clean-up technology and its performance on soils collected from Raša coalmines and to examine the efficiency of the bioremediation method applied for the first time on Labin environmental samples. Accordingly, specific objectives have been set:

- to isolate and determine species of bacteria from the soil samples that will be used for the cleaning treatment
- to measure concentration of sulfur after carried out treatment
- to determine and compare initial concentration of sulfur in the untreated samples with final measurements
- to determine the correlation between sulfur reduction and duration of exposure to bacteria.

2. RESEARCH AREA

2.1. Historical review of Istrian coalmine Raša

Istrian coalmines Raša was a mining company which after the World War II involved several mines (Vinež, Tupljak, Labin, Ripenda, Raša, Koromačno) in a joint production. Permanent mining production in Labin started in 1830s owned by a stock company Adriatische Steinkohlen-Gewerkschaft in Dalmatien und Istrien. The mines were modernized in 1881 by company Trifailer Kohlenwerks-Gesellschaft which connected mines in Labin into one manufacturing technology. Compressed air was introduced for the launch of mining machinery and locomotives replaced the horses. At that time, the number of workers exceeded one thousand and the annual production was between 90 and 130 thousand tons of coal. In the 1930s, the Raša coalmines belonged to Italian company under which management sudden growth of production was recorded due to the need of a fascist economy and Raša coalmines started to be the most modern ones in Europe. The increase in number of employers (more than 10000) and in production (in 1942 reached more than one million tons) can be contributed to modernization of mines, especially in electrification. On 28th of February 1940 occurred the explosion of methane where 372 miners were killed. After the World War II Raša coalmines became very important for economic recovery and reconstruction of Yugoslavia. In the 1960s, cheap oil caused a coal crisis. Large coal reserves caused a decrease of production and the number of workers while thermal power plant Plomin 1 was built to reduce the stock. Nonetheless, the mines survived the crisis and then began the construction of another thermal power plant. The coalmine in Labin was closed in 1988 and in Tupljak in 1999 which was the last coalmine in Croatia (Vorano, 1997; Fonović 2000).

2.2. Raša coal characterization

Raša coal belongs to stone coals. It is black, shiny, burns long and it has high caloric value from 25000 to 31000 kJ per ton. This coal contains 65-75 % of carbon and amount of sulfur can be up to 14 %. Due to its high sulfur amount, it is classified as superhigh-organic-sulfur (SHOS) coal. Carbon ash and its byproducts of combustion also show elevated Ca, Mg, Se and

U concentrations which are characteristic elements of seawater. This fact shows that the layers of coal are closely related to the sea carbonate layers (Istarska enciklopedija, n. d.; Medunić et al., 2016a).

2.3. Geographical characteristics of Istria

Istrian Peninsula is the biggest Croatian peninsula with the area of 3 476 km². It is situated on the northern part of the Adriatic Sea. Most of its territory belongs to Croatia but northern parts are bordered by contiguous Slovenia and Italy. Based on a geological compounds and different types of soils, Istria is divided in three parts (Figure 5). The White Istria is mountain part consisted of karst and includes the north of peninsula (Učka i Ćićarija). Inner fertile terrain is called the Grey Istria, also known as the Flisch Istria. The main characteristic is erosion-lowered watertight layer of flisch with high content of clay. The Red Istria is situated on the littoral part of the peninsula. The name 'Red' was given due to terra rossa, type of soil that originates as product of abrasion of limestone (Hrvatska enciklopedija, n. d.).

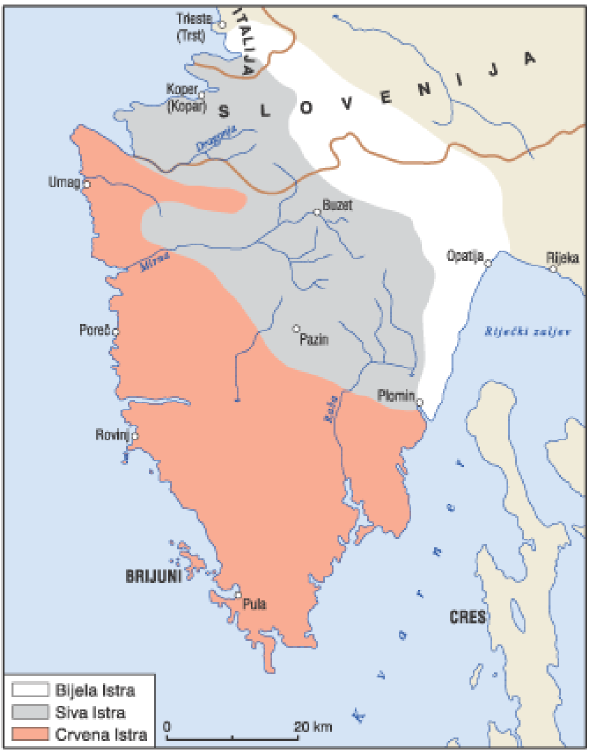

Figure 5. Geomorphological division of Istria.

(izvor: http://istra.lzmk.hr/)

2.4. Climate of Istria

Very important moderator of Istrian climate is the fact that she is surrounded with sea from three sides. Mediterranean type of climate, which dominate along the coast, is gradually changing to temperate warm due to nearness of Alps. The northeast parts are characterized with mountain climate conditions while coastal zone has the highest temperatures and the lowest total rainfall. East side of peninsula has warm humid climate with hot summers while in the inside part prevails more temperate with warm summers. Average temperature of air per year is 14 °C along the north part of the coast and on south it amounts 16 °C. The coldest month is January with temperatures around 6 °C whilst the hottest month is August with mean temperature of 24 °C. The lowest temperature of the sea is in March, between 9 and 11 °C while the highest is during August with the value of 24 °C (Filipčić, 1992).

The amount of rainfall is getting bigger from west side to inside of the peninsula as consequence of impingement of air masses between two relief barriers Slavnik and Učka. Due to lift of air along the barrier side, condensation and precipitation starts to occur. Therefore, the northwest highland is the moistest part with above 1500 mm of rainfall per year. The northwest of the coast has between 900 and 1100 mm of rainfall per year while the rest of coastal area is characterized with total rainfall between 800 and 900 mm per year. The hole peninsula has the same regime of rainfall with the highest rainfall during the October and November. There also exists secondary extremity on the transition from spring to summer while the end of the winter, beginning of the spring and summer have the lowest amount of rainfall (Filipčić, 1992).

The characteristic winds are bora, jugo and mistral. The bora blows from the northeast and east. It's most common during the winter due to period of low pressure above the Adriatic Sea. The anticyclonic bora brings clear sky and cold weather while cyclonic bora can bring abundant rain or snow. In colder period of the year, along with bora can appear warm and moist jugo blowing from the southeast. Cloudy and rainy weather can be present during the cyclonic jugo. At stable and clear weather, from sea toward the land winds refreshing and mild mistral (Filipčić, 1992).

According to Köppen classification, Istria is characterized with climates from C class. Cfa (temperate humid climate with hot summers) and Cfb (temperate humid climate with warm summers) are present (Šegota and Filipčić, 2003).

2.5. Geological characteristics of Istria

Istrian peninsula makes a northwestern part of an old Adriatic carbonate platform. On that large paleogeographic body, under shallow and warm sea, carbonate rocks had been precipitated. Platform had been isolated from terrestrial influence due to surroundings of Tethys ocean for long period, from lower Jurassic (before approximately 190 million years) until the end of Cretaceous (before approximately 65 million years). Under that conditions, large deposits of carbonate had been arising. The depth of deposit is around 500 m and most of it are limestones with few dolomites. Nowadays, the biggest part of platform is under Adriatic Sea (Vlahović et al., n. d.).

Four sedimentary units are present on Istrian peninsula. Three of them are deposits of inner part of Adriatic platform and fourth one is made during its disintegration. Its surface is mainly covered with relatively thin layer of quaternary deposits. The most important tectonic deformations are west-istrian anticline arisen in Cretaceous and tertiary forms such as thrust structures of Učka and Ćićarija (Figure 6). The continuum of sedimentation had been interrupted with few terrestrial phases (Vlahović et al., n. d.).

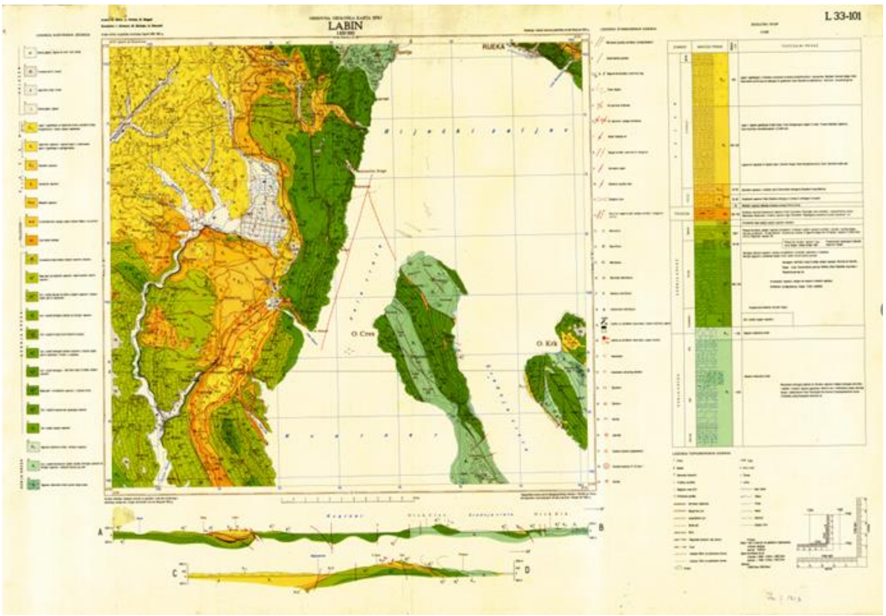

Figure 6. Basic geologic map, 1 : 100 000 L 33 – 101 Labin.

The primus sedimentary unit, unit I, includes deposits from Middle Jurassic (Bathonian, before 170 million years) to Late Jurassic (Kimmeridgian, before 155 million years). Typical rocks are shallow water marine limestones containing numerous fossils such as corals, shellfishes, hydrozoa etc. The second sedimentary unit, unit II, comprises deposits from Late Jurassic (Tithonian, before 150 million years) to Early Cretaceous (Aptian, before 115 million years). After progressively flooding of former land, limestone had been precipitated in tidal plains. Due to alternation of depositional conditions from extremely shallow to rather deeper, huge amounts of limestone silt and algal clods had been deposited. The sedimentation was discontinued approximately before 115 million years during Aptian. The terrestrial phase is observable in sequence for appearance of one-meter thick layer of breccia. The third sedimentary unit, unit III, includes deposits from Early Cretaceous (Albian, before 110 million years) to different ages of Late Cretaceous. Due to unequal finish of sedimentation, deposits pertaining to this sedimentary unit have different depth. Interesting is that among layers of limestone exist layer of volcanic ash which can be attributed to volcanic activity nearby. This deposit is original material for quartz sands. The fourth and last sedimentary unit, unit IV, consists of Paleogene deposits whose sedimentation had begun with flooding of karstified land. Parallel with the elevation of sea level was also elevated groundwater level. Consequently, the most lower parts were transformed into wetlands.

Under that conditions plant material, that had later become coal (coalmines in Labinština), had been deposited. To this unit belong deposits of foraminifera limestone and flisch. Flisch contains marl in lowest parts (mixture of fine-grained particles of calcite and clay) and regular alternations of autochthonous deposits and calcareous sandstone. Before approximately 35 million years all area of Istrian peninsula had become land. During such a long terrestrial period, all four sedimentary units had been exposed to erosion and karstification. The youngest sediments are terra rossa, loess, quaternary breccia and wetland sediment (Vlahović et al., n. d.).

The tectonic activity on Istrian part of Adriatic carbonate platform can be divided in few active and structure-recognizable periods. The oldest period is related to Cretaceous when the platform has direction west-northwest. Due to resistance, new formed structures have orientation north-northeast-south-southwest like west-istrian anticline. During the Paleocene, the moving direction of the platform was rotated in northeast-southwest. As a result, rising of Dinarids has begun and all the ‘dinaridic’ structures as thrust structures of Ćićarija and thrust fault of Učka. In the late Miocene begins the neotectonic period which is still present nowadays. Due to north-south compression, structures have direction east-west (Vlahović et al., n. d.).

2.6. Soil

Soil is biologically active and porous surface layer on Earth’s crust. It’s one of the most important substrata of life serving as a source of nutrients for plant production and other organisms. It has certain role in regulation of chemical composition of the atmosphere in lower layers, hydrosphere and biological circulation of matter in nature. With climate it controls the distribution and number of living organisms in ecosystem. It acts as a filter and buffer in physical, chemical, physicochemical and biochemical processes in soil. Soil is the base for all human labor and a source of raw materials (Husnjak, 2014).

Factors of the soil formation under which they are created and developed are: parent material (lithosphere), living organisms, climate, relief, hydrological conditions, man's activity and time (Figure 7). Similar conditions cause same or similar type of soil. Soil is four-phased system consisting of solid phase, solution, air and living organisms. Solid mineral phase makes

30 to 60 % of it and exist few categories of particles depending on their size. If size of a particle is less the 0,002 mm, it is classified as clay, or as a silt if the diameter is 0,002 – 0,05 mm, or as a sand if the size is 0,05 – 2 mm and colloidal particles if the size is above 2 mm. Those mechanical elements are usually bound in clusters called structure aggregates. They affect the soil ability to retain and transport water, air and nutrients. Crumb structure, where aggregates are rounded and 2 – 10 mm in diameter, is ecologically and plant-productionally most welcome. The spaces between structural aggregates are called pores. In terms of size and function, capillary pores for water and noncapillary pores for air may differ. From the latter the root system of plants is supplied by oxygen. The water-air ratio is varied depending on amount of precipitation, water penetration and evaporation/transpiration. The physicochemical and biochemical processes are strongly influenced by the relationship between water and air in soil. The liquid phase is mainly composed of water in which different inorganic and organic substances are dispersed or dissolved. The concentration of biogenic elements (N, P, K, Ca, Mg and others) and hydrogen ions (pH) that can be found in this phase are very significant for plant production (Husnjak, 2014).

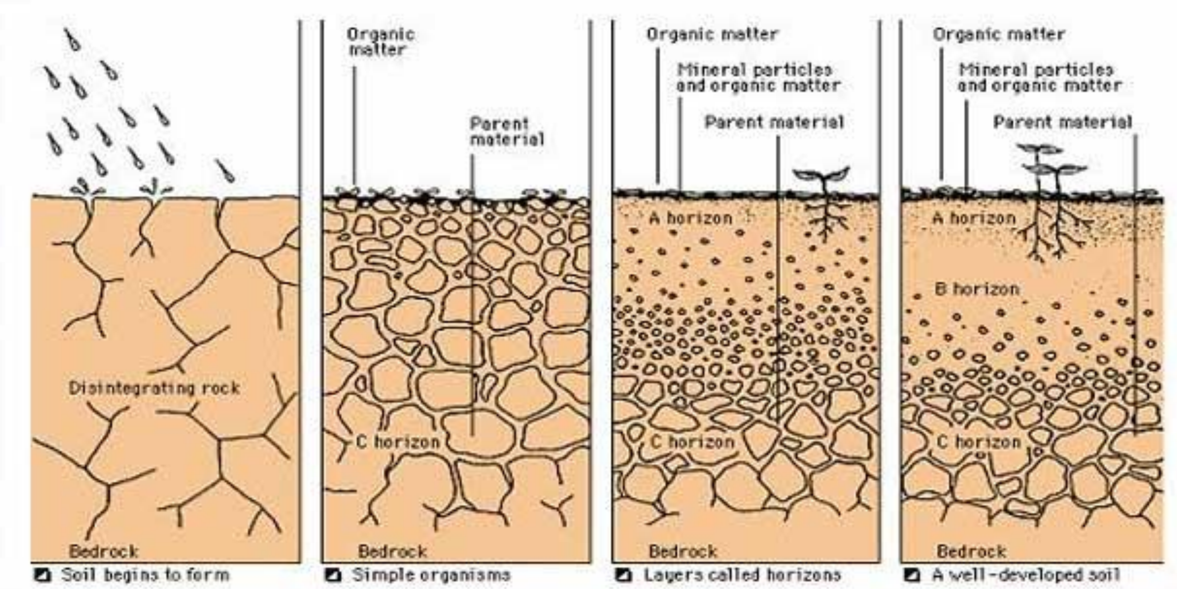

Figure 7. Soil formation.

(http://www.civileblog.com/formation-of-soil/)

All soils have a unique structure made of layers called horizons and the full vertical sequence of horizons makes the soil profile. Soil horizons are defined by soil-forming processes and its features. A-horizon is a surface humus-accumulative horizon with microbial biomass that is mixed with small-grained minerals to form aggregates. E-horizon is an elusive horizon developed in conditions of high rainfall and sandy parent material, two factors that ensure water percolation. The particles washed through leaching are deposited in the B horizon. B-horizon is regarded as a zone of illuviation. It is characterized by an accumulation of clay deposited out of percolating water. C-horizon is unconsolidated parent material from which all above mentioned horizons are made. Almost no soil development or humus accumulation are present. R-horizon represents consolidated rock. O-horizon represents uppermost organic layer consisting of more than 35 % of organic matter. G-horizon is gleaning horizon and its development is favoured by anaerobic conditions. It shows reductions and secondary oxidation. P-horizon is anthropogenically evolved horizon which affect at least A-horizon (Encyclopaedia Britannica, n. d.).

Soil degradation is decline is soil quality where the balance of soil natural resistance has been disturbed by environmental conditions or human activity (Oldman, 1992). Pathways of soil degradation are numerous such as intake of pollutants from atmosphere, destruction of soil structure, water and wind erosion, entry of organic pollutants through agricultural activity, increase in salinity, loss of organic matter, soil acidity or alkalinity, mass movement and soil contamination with toxic elements and pollutants (Soil degradation, 2018).

2.6.1. Soils of Istria

Thanks to the great variability of soil-forming factors, a large number of genetic and ecological contrasting soils were created in our area. In Istria exist 17 types of different soils (Gluhić, 2005). According to Systematics of soils of Croatia (Husnjak, 2014) they can be categorized in several classes:

1. Initial terrestrial soils: colluvial soil
2. Humus-accumulative soils: melanosol (leptosol on hard limestone and dolomite), rendzina, vertisol

3. Cambic soils: brown soil, cambisol on limestone and dolomite, terra rossa
4. Eluvio-illuvial soils: luvisol, akrisol
5. Amphigley soils: pseudogley, pseudogley-gley
6. Anthropogenic soils

and combinations. Among these, on peninsula of Istria the most common are terra rossa, rendzina and cambisol on limestone and dolomite covering almost 80 % of Istrian area (Gluhić, 2005).

3. MATERIALS AND METHODS

3.1. Soil sampling

The soil sampling was begun with the removal of the vegetation cover. 1 – 2 kg of topsoil were taken with shovel. The taken samples were air-dried, gently disintegrated and then sifted through the net with 1 mm-sized pores. Thereafter, homogenization, weighing and analyses were followed. Samples were collected in September of 2016 and locations are presented in Figure 8.

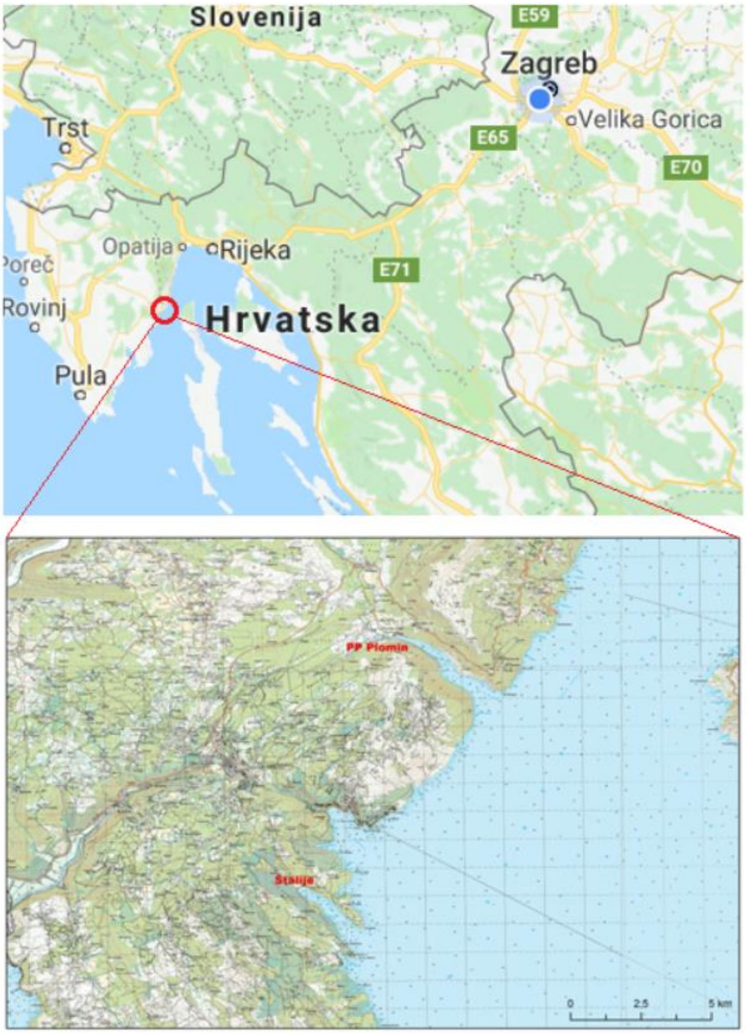

Figure 8. Map of locations.

(https://www.google.com/maps; Topographic map of Croatia 1 : 25000 - https://geoportal.dgu.hr)

Description of locations:

- thermal power plant Plomin 1

  By the beginning of the 1990s, coal from Croatia was used (anthracite from Istria and others) with high sulfur amount (10 to 14 %) resulting in highly enriched deposit of slag and ash Towards the end of the 1990s, reparation began with application of protective foils, covering the soil and construction of channels and collectors for the drainage of waste water. Later was Raša coal replaces with imported one with lower sulfur amount from 0,3 to 1,4 %. Nowadays, the waste produced in the power plant is suitable for use in construction and cement industries (Klapčić, n. d.).

- port Štalije

  Štalije is one of the ports of Raša river. The port was used for the export of coal until the end of 1960s. Nowadays, the port is used for the transshipment of bulky materials (Fabjanović, 2005).

3.2. Bacterial bioremediation

The samples are treated with a known bacterial species, *Ralstonia sp*. which has been grown, separated and mass cultivated under optimum conditions (temperature of 35^{o}C and pH 7). Bacteria was identified by the Institute of Microbial Technology (IMTECH), Chandigarh (India).

3.2.1. Chemicals and laboratory equipment needed for analysis

List of chemicals and laboratory equipment needed for analysis:

- laminar flow cabinet
- autoclave
- gas burner
- beakers
- watch glasses

- Petri plates
- Erlenmeyer flasks
- inoculation loop
- insulation tape
- glucose, anhydrous
- peptone, bacterial
- yeast extract powder
- agar powder
- sodium alginate ($NaC_6H_7O_6$)
- calcium chloride ($CaCl_2$)
- methanol
- hydrochloric acid (HCl)

3.2.2. Procedure

a) isolation of bacteria and mass cultivation

Every used laboratory glassware for the experiment was sterilized with hydrochloric acid. 300 mL of solid and 300 mL of liquid media were prepared in Erlenmeyer flasks as mentioned underneath:

- solid media: 300 mL of double distilled water, 0,5 % peptone, 0,5 % yeast extract, 1,0 % glucose, 3,0 % agar

- liquid media: 300 mL of double distilled water, 0,5 % peptone, 0,5 % yeast extract, 1,0 % glucose.

All components were put in Erlenmeyer flasks, closed with cotton plugs and paper, and put into autoclave for one hour on 120 °C and pressure of 1 bar. After autoclaving, solid media was divided and put into few Petri plates before cooling of the solution. The work was done in the laminar flow cabinet. The working area was first cleaned with methanol. Gas burner was on and hands were washed with methanol. Every work was done near the fire.

The plug from the flask was taken and the neck of the flaks was burned with flame. After media was put in Petri plates, each plate was closed with insulation tape.

1 gram of each sample was weighed and mixed with 10 mL of double distilled water in the beaker. Few drops of the solution were inoculated on solid media in zig-zag form using inoculation loop following the same sterile procedure. Inoculation loop was, before using, heated until becoming red, put in methanol and under the flame. Within 24 hours from inoculation (Figure 9), bacteria were picked up from Petri plates and transferred to test tubes with 2-3 mL of liquid media (Figure 10). After three days, content of each test tube was transferred in conical flasks with 50 mL of liquid media inside.

Figure 9. Bacterial growth after 24 hours from inoculation.

Figure 10. Transferring bacteria from Petri plates to test tubes.

b) immobilization of bacteria

The exponential phase bacterial cells (1000 mg) was representing the growth phase of bacteria where bacterial cells were dividing as fast as possible by intake of nutrients from culture media. The exponential phase was obtained through centrifugation at the rate of 10000 rpm for 10 minutes and washed two times with double distilled water to remove any sort of contamination, microorganisms and extract of growth media. The resultant bacterial biomass was then mixed into 100 ml of 5% (w/v) solution of sodium alginate ($NaC_6H_7O_6$) prepared in the glucose-peptone-yeast (GPY) medium. The mixture was pumped drop wise into 0.2 M $CaCl_2$ solution in the laminar flow cabinet, which acted as a gelling agent to provide more stable beads. The beads, thus formed, were harvested and resuspended in a 100 ml growth medium (GPY) contained in 250 ml cotton plugged culture flask (Figure 11).

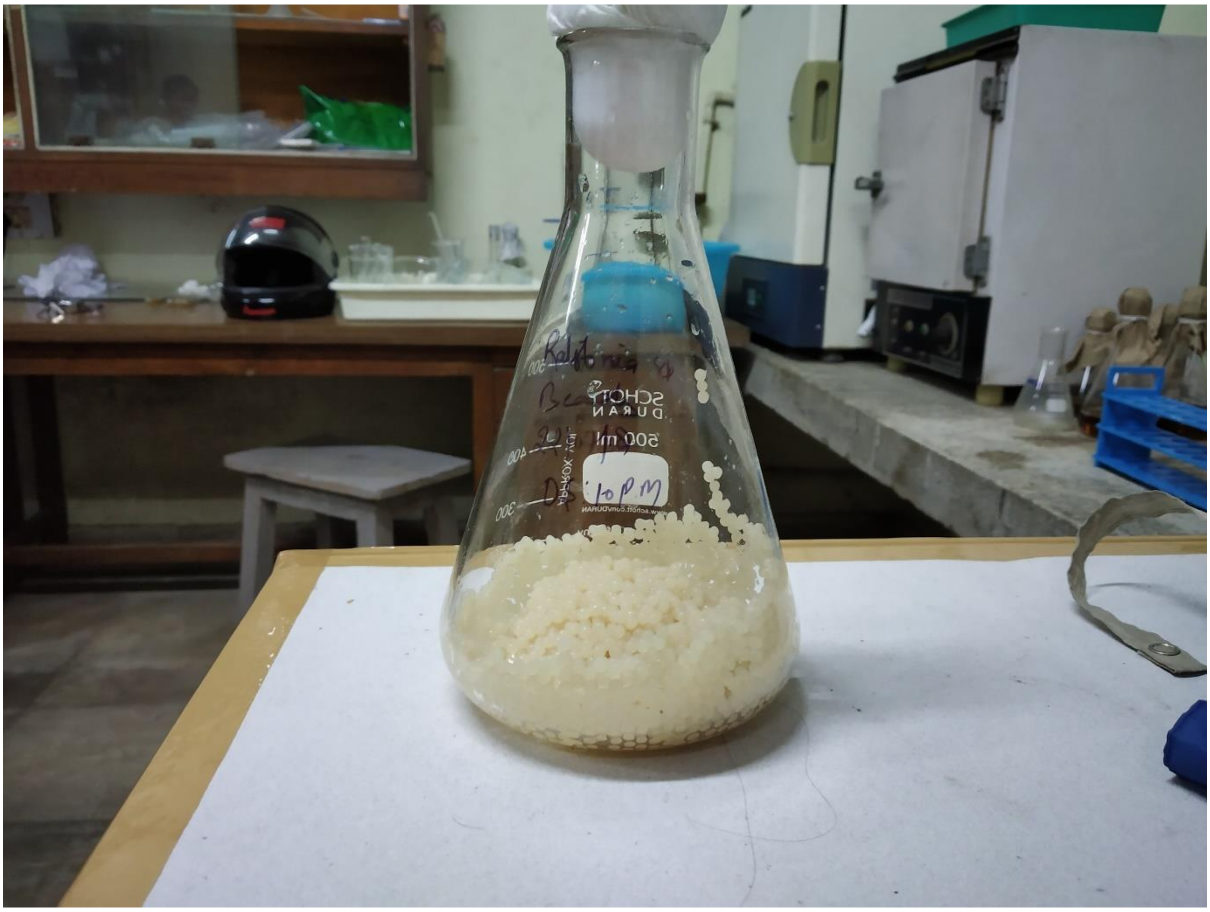

Figure 11. Bacterial beads.

c) treatment of the samples with bacterial beads

The samples were initially sterilized under ultraviolet rays for up to 45 minutes in the laminar flow cabinet before treatment, so they were free from any microorganisms. 5.0 gram of each sterilized sample were put in the conical flask containing 75 mL of sterilized distilled water. Thereafter, 100 beads* (equivalents to 1000 mg dry weight of bacteria) were added in each flask. The treatment was carried out for seven days and the total sulfur concentration has been determined after first, third, fifth and seventh day.

*It was calculated that from 1 mL solution of biomass and sodium alginate, 25 beads were formed. So, for 100 beads, 4 mL of solution was required. And likewise, it was calculated that 250 mg of dry weight of bacteria were contained in 1 mL of the solution of bacterial biomass in sodium alginate. Therefore 4 mL solution, which was required for 100 beads, contained 1000 mg of dry weight of bacteria.

3.3. Gravimetry

Standard test method for total sulfur amount in the sample of refuse-derived fuel was carried out. It is Eschka method where the weighed sample and Eschka mixture were ignited together and the sulfur was precipitated from the resulting solution as barium sulfate ($BaSO_4$).

3.3.1. Chemicals and laboratory equipment needed for analysis

List of chemicals and laboratory equipment needed for analysis:

- cold muffle furnace
- gas burner
- porcelain crucibles
- funnels
- filter paper
- graduated cylinders
- beakers (200 mL, 600 mL)

- barium chloride solution, $BaCl_2 \times 2H_2O$ (100 g / L)
- bromine water, saturated
- Eschka mixture – two parts of magnesium oxide (MgO) and one part of anhydrous sodium carbonate (Na_2CO_3)
- hydrochloric acid (1 + 1; 1 + 9)
- methyl orange indicator solution (0,2 g / L)
- sodium hydroxide solution (100 g / L)
- distilled water

3.3.2. Procedure

Samples were dried in oven at 105 °C for 4 hours. Porcelain crucibles were heated over a gas flame, each for 5 min. 1 g of each sample was weighed. Eschka mixture was weighed two times, one set had 2 g of the mixture and other 1 g. Samples and 2 g of the mixture were mixed thoroughly. Quantitatively were transferred to a porcelain crucible and covered with 1 g of Eschka mixture. Crucibles were placed in a cold muffle furnace and heated to 800 °C for about 1 hour.

Crucibles were removed from the furnace, cooled and replaced quantitatively in 200 mL beakers. Samples were digested with 100 mL of hot water for 40 min with occasional stirring. The supernatant liquid was decanted through a filter paper into a 600 mL beaker. The insoluble matter was washed with hot water several times. After washing, the insoluble matter was transferred to the filter paper and wash again five times with hot water. All wash water was collected in 600 mL beaker. The filtrate was treated with 15 mL of saturated bromine water and made slightly acid with around 2 mL of concentrated HCl and boiled to expel the liberated bromine. after the boiling, it was neutralized using methyl orange indicator with NaOH (from red-coloured solution to yellow) and after was added 1 mL of HCl solution (1 + 9). It was boiled again while stirring constantly. 10 mL of $BaCl_2$ solution was added slowly into the solution. It was boiling for 15 min more, covered with watch glass and put to stand overnight at ambient temperature. The solution and the precipitate of barium sulfate ($BaSO_4$) were filtrated through filter paper (Figure 12).

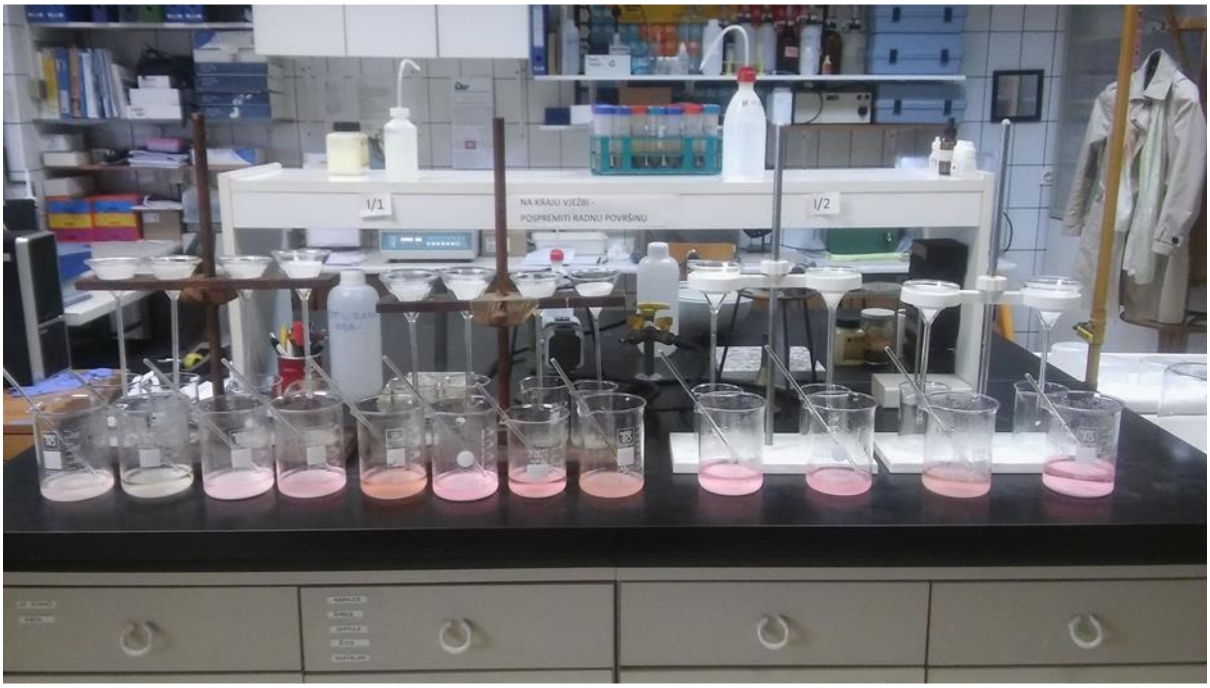

Figure 12. Filtration of solution containing $BaSO_4$.

The $BaSO_4$ residue was washed with hot water until one drop of silver nitrate ($AgNO_3$) solution produced no more than a slight opalescence when added to 10 mL of the filtrate. The wet filter paper containing the precipitate of $BaSO_4$ was placed in porcelain crucible allowing a free access of air by folding the paper over the precipitate loosely to prevent spattering. Porcelain crucibles were heated over a gas flame, each for 5 min and weighed. The paper was replaced in crucibles and smoked off gradually over a gas burner (figure). After charring of the paper, crucibles were replaced in muffle furnace and heated to 925 °C for 3 hours (Figure 13). The crucibles were transferred to a desiccator and weighed when cooled to room temperature.

Figure 13. A – smoking the filter paper with precipitated $BaSO_4$ over a gas burner; B – porcelain crucibles with precipitated $BaSO_4$ in cold muffle furnace.

3.3.3. Calculation

The sulfur amount was calculated as followed:

$$\text{sulfur, \%} = (A - B) \times 13{,}738 / W$$

where:

A = grams of porcelain crucible + precipitated $BaSO_4$

B = grams of porcelain crucible

W = grams of used samples

13,738 = percentage of sulfur in $BaSO_4$.

4. RESULTS

The measured percentages of sulfur in three samples are shown in Table 1. Sample Štalije and Plomin are samples of soil and Coal is coal sample. Plomin is the sample with the lowest sulfur amount (from 1,19 to 1,23) while coal is with the highest (from 10,19 to 10,65).

Table 1. Sulfur amount in % in two samples of soil and one coal sample.

	Štaljie	Plomin	Coal
1st day	3,0113	1,2254	10,6467
3rd day	3,2235	1,2039	10,5429
5th day	3,0173	1,1900	10,1401
7th day	3,2484	1,2176	10,1926
average	**3,1251**	**1,2092**	**10,3806**

Sulfur amount of soil sample Štalije is showing that percentage of S on 1st day (3,01 %) is lower when compared to 7th day (3,25 %). amount of S from 1st day to 3rd day (3,22 %) is increasing, from 3rd day to 5th day (3,02 %) is decreasing and eventually in the last third is increasing again. The lowest value of sulfur is on 1st day and on 7th is the highest. This increasing-decreasing trend in total sulfur amount caused increasing trend line (Figure 14).

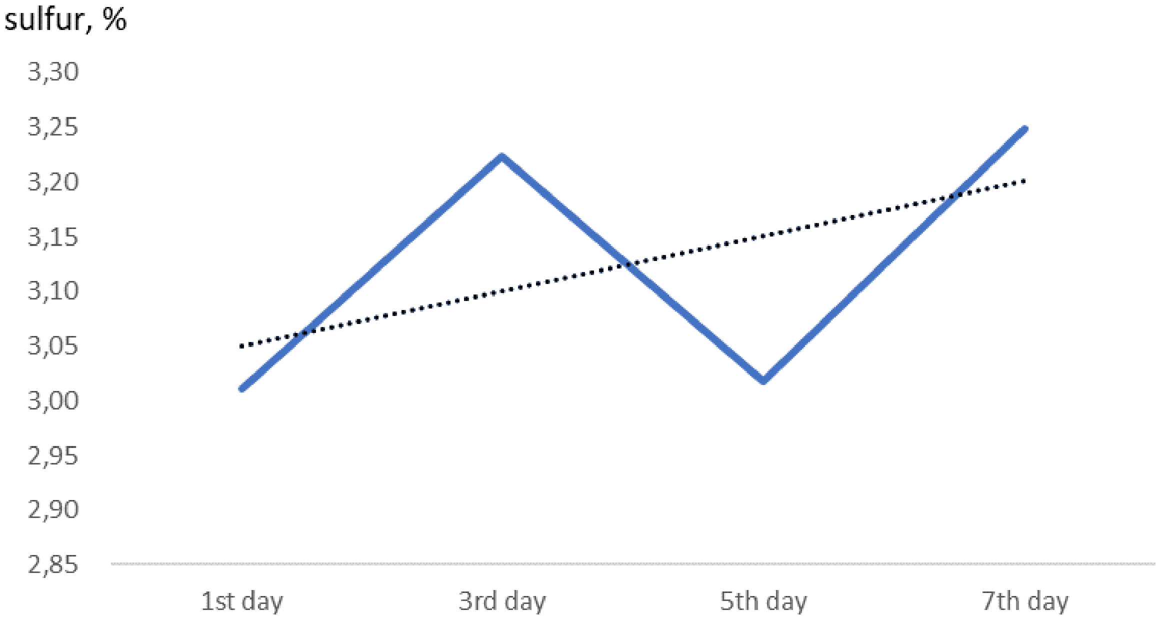

Figure 14. Sulfur amount of soil sample Štalije during 7 days with trend line.

Sulfur amount of soil sample Plomin is showing that percentage of S on 1st day (1,23 %) is higher than percentage of S on 7th day (1,22 %). Sulfur amount from 1st day until 5th day is decreasing and in the last third is increasing. The lowest value of sulfur is on 5th day (1,19 %) and the highest on 1st. Total trend line is decreasing (Figure 15).

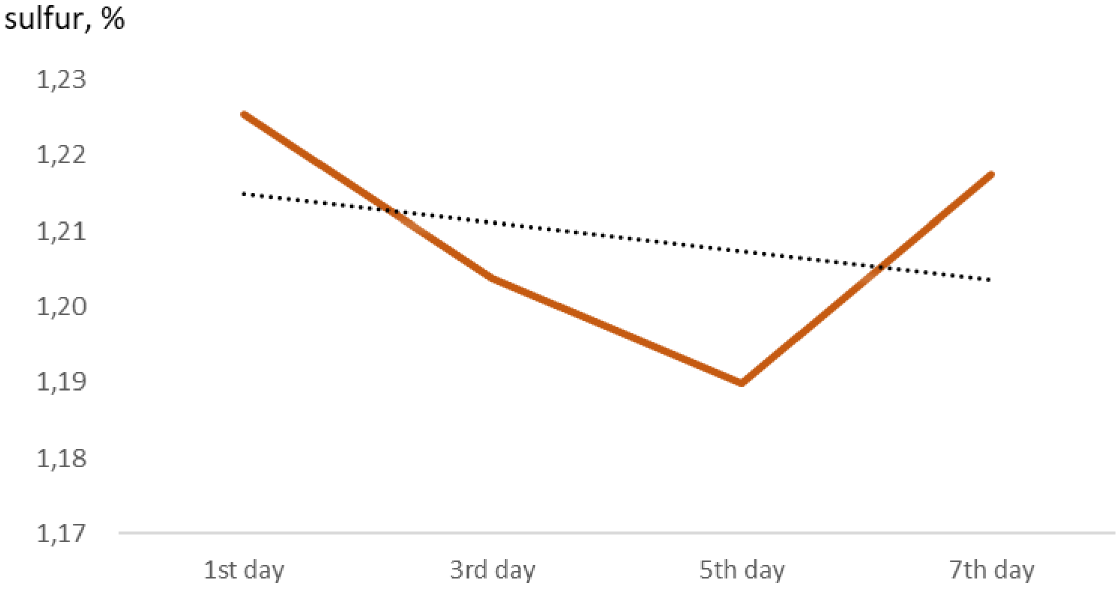

Figure 15. Sulfur amount of soil sample Plomin during 7 days with trend line.

Sulfur amount in coal sample is showing that percentage of S on 1st day (10,65 %) is higher than percentage of S on 7th day (10,19 %). Sulfur amount from 1st day until 5th day is decreasing and in the last third is increasing. The lowest value of sulfur is on 5th day (10,14 %) and the highest on 1st. Total trend line is decreasing (Figure 16).

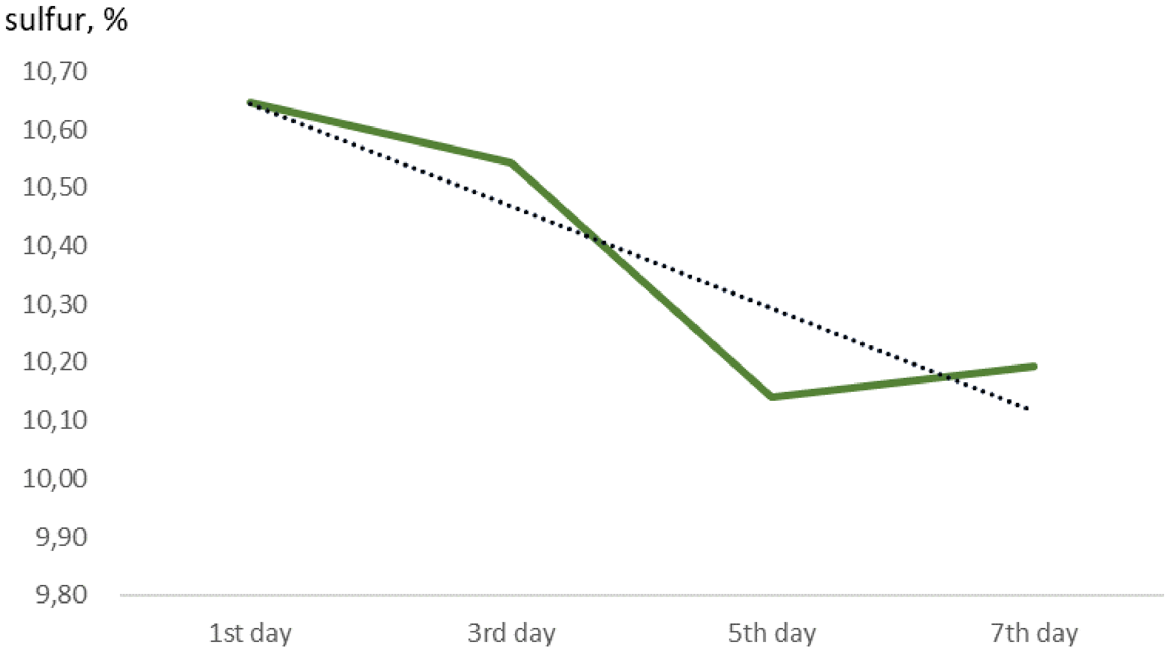

Figure 16. Sulfur amount of coal sample during 7 days with trend line.

5. DISCUSSION

Previous investigations provided by Medunić et al. (2016a) showed that initial values of total sulfur (S_t) in Raša coal were around 11 %, in the sample Štalije around 6 % and in the sample Plomin around 4 %. Initial values of sulfur in those samples are in accordance with air distance of localities Štalije and Plomin where the second one is 20 km distant from the first locality. Results are showing significant decrease of S_t compared to initial values. In the sample Štalije removal of sulfur is 47,83 %, in Plomin 69,75 % while in coal sample it is relatively low removal of 5,64 %.

5.1. Desulfurization depending on sulfur species and coal rank

The different species of sulfur in coal are closely related to the depositional conditions of coal. Depending of sulfur amount in coal, exist low-sulfur (< 1 % of S), medium-sulfur (1-3 % of S), high-sulfur (3-4 % of S) and superhigh coal (4-11 % of S) (Chou, 2012; Medunić et al., 2018). Results given by Medunić et al. (2018) show that superhigh-organic-sulfur (SHOS) Raša coal has extremely high amount of sulfur, up to 11,8 % which is mainly present in form of organic sulfur (11,4 %). The dispersion of $S0_2$ and ash particles around the thermal power plant Plomin caused contamination of nearby soils with sulfur and PAHs (Medunić et al., 2016a). The main source of this extremely high amount of organic sulfur in Raša coal wasn't the parent material (Medunić et al, 2018). Therefore, the cause must be found in another source. According to Chou (2012) different amount of sulfur suggests different sources of it in the coal. For low-sulfur coal, sulfur originates primarily from parent plant material but for medium-, high-, and superhigh-sulfur coal, two sources are main: parent material plant and sulfate in seawater. Sulfate is under certain environmental conditions (like in seawater) reduced to hydrogen sulfide (H_2S) and elemental sulfur by bacteria (Chou, 2012; Jørgensen, 1977). The incorporation of sulfur into organic matter is favoured by an alkaline marine environment with high amount of hydrogen sulfide (Medunić et al., 2016b).

The uptake of sulfur-containing compounds is selectively made by bacteria. Thus, type of sulfur species present in the coal is affecting quite a lot the efficiency of bacterial bioremediation (Berthelin, 1988). The sulfur-containing compounds in coal can be present as both aliphatic sulfur-containing chains (mercaptans, aliphatic sulfides, thiophenes) and heteroatoms in aromatic rings (Calkins, 1987; Rađenović, 2004). In high-sulfur coals the relative proportion of aliphatic structures to total organic sulfur appears to be in the range of 30 - 50 % (Calkins, 1987). Organic or pyritic sulfur amounts may depend on coal rank where higher coal rank has higher amount of labile sulfur-containing compounds such as aliphatic thiols and sulfides (Boudou et al., 1987; Rađenović, 2004). High molecular weight compounds such as aromatic are usually more stable and therefore, heavier to break by bacteria (Boudou et al., 1987; Wrenn and Venosa, 1995). Accordingly, it could be suggested that Raša coal with its high rank and high sulfur amount can have more sulfur-containing compounds in form of aliphatic structures which would facilitate bacterial desulfurization, but this is yet to be investigated.

Sulfur is present in all macerals and most minerals where vitrinite contains the major part of organic sulfur and metals (Rađenović, 2004). Results carried out by Singh et al. (2012) are showing that from four investigated soils, decrease of total sulfur showed a strong positive correlation with inorganic matter, hydrogen content and negative correlation with macerals (vitrinite, inertinite, liptinite).

Desulfurization rate was observed to increase in samples with lower initial total sulfur values and higher desulfurization in samples having relatively low quantity of sulfur (Singh et al., 2012). This is in accordance with obtained results from Raša coal and two soils samples. The sample Plomin, having the lowest initial S_t, has the highest removal percentage. The coal sample with the highest initial S_t showed the lowest removal.

5.2. Bioremediation depending on bacteria

Bacteria used for bioremediation treatment must fulfill some requirements such as high bioremediation capacity and low or none negative impact on the environment. Bacterial cultures can remove much of the inorganic sulfur, but the organic is much more difficult to remove (Acharya et al., 2001). Raša coals are high with organic sulfur so adequate bacteria must be used. Further, exist potential possibility of having negative impact on ecosystem. Some bacterial species like in gender *Pseudomonas*, although observed as having quite high relatively bioremediation capacity, caused environmental problems where marine or river organisms showed especially sensitive (diseases and fin rot in fish) (Ryan et al., 2007). So, the use of such bacteria is not advisable. *Ralstonia sp.* showed many advantages for the cleaning treatments due to its large range of environmental conditions, biodegradative abilities and large metabolic diversity (Ryan et al., 2007). *Ralstonia sp.* has been used also for the bioremediation of toluene from groundwater in South Carolina (Vroblesky et al., 1997). Measurements made on Indonesian and Indian soil where the same bacterial species was used showed removal from 5,81 % up to 68,66 % (Singh et al., 2012). Results from other investigations using other bacterial species showed the sulfur removal of 27,20 %; 50 %; 31-51 %; 13,23 % (Tripathy et al., 1998; Aller et al., 2001; Cardona and Márquez, 2009; Singh et al., 2015).

Results from the two investigations on the same Indian soil sample, comparing desulfurization capacity of two bacteria *Pseudoxanthomonas sp.* and *Ralstonia sp.* (41,84 % - 45,50 %; 18,26 % - 11,83 %; 34,16 % - 20,22 %) show that more successful in the removal is *Pseudoxanthomonas sp.* (Singh et al., 2012; Singh et al., 2013).

The environmentally sensitive elements are likely to cause environmental problems. The potentially toxicity of the trace element is related to their mobility in environment. Therefore, they are required to be removed or minimized (Singh et al., 2015). Bacterial removal of metals from coal is rendered through leaching, adsorption and accumulation. The leaching takes place in several ways such as redox reactions, formation of organic or inorganic acids and excretion of complex compounds (Berthelin, 1988). Adsorption occurs on the bacterial surface. Due to nature of bacterial capsule or cell wall where numerous negatively charged compounds can be found, positively charged metal (cations) make ionic bonds with them. Translocation of accepted cations is referred as accumulation. In this metabolic depended process which involves often special channels or pores on cell membrane, bacterial removal of metals is finished (Gadd, 2000). High occurrence of organic sulfur and metals in vitrinite suggests the presence of organosulfur-metallic species (Boudou et al., 1987). Due to relatively successful desulfurization by bacteria, further investigations on superhigh-organic-sulfur Raša coal should be carried out to see the effect bacteria have on reduction of potentially toxic and trace metals in the same coal.

Until now there have been some investigations about certain strains of *Ralstonia sp.* Uttukar et al. (2013) suggested that strain OR214 is tolerant to high concentrations of heavy metals such as up to 50 M of cadmium, 50 M of cobalt, 500 M of nickel, 200 M of uranium and 500 mM of nitrate that make this strain highly suitable for bioremediation treatment. The genome sequence is affecting several physiological advantages as heavy metal translocating efflux pumps, heavy metal-sensing proteins and monooxygenase is potentially involved in the breakdown of aromatic compounds. Further knowledge about which genome sequences are responsible for successful treatment will contribute toward better bioremediation practices.

5.3. Bioremediation depending on time, bacterial biomass, temperature and coal particle size

It was shown that with longer time the samples were exposed to bacteria, the removal of S_t was also increasing. However, after certain time very little decrease of sulfur amount was observed (Tripathy et al., 1998). Tripathy et al. (1998) and Acharya et al. (2001) got to conclusion that after 15th day removal of sulfur in slowing down. This could be due to the consumption of all available sulfur to bacteria or connected with the decline phase of bacterial growth. Another important observation is that in first few days the removal of sulfur is present but is relatively slow (Tripathy et al., 1998; Singh et al., 2016). That phenomena can be explained with lag phase of bacterial growth where bacterial cells need to adopt to new conditions and where no cell division is present. So, it’s very advisable for higher efficiency to determine exponential phase of bacteria where the best results in removal percentage can be expected.

When larger bacterial biomass is used for the bioremediation, significant increase in the removal of elements is observed. Singh et al. (2016) compared results of minor and trace elements removal with 10 mg/mL and 25 mg/mL of bacterial biomass. Minor (Ca, K, Na) and trace elements (Ni, Zn) treated with 10 mg/mL of biomass showed reduction of the same but in lower rate compared to 25 mg/mL. Therefore, the same principle may be applied for the sulfur removal as a function of change in the concentration of bacterial biomass.

The bioremediation process can also be a function of temperature. It is noticed that for a mixture of bacteria used for the treatment, the maximum degree of desulfurization was obtained between 30 and 35 °C where at higher temperatures the removal is significantly decreased (20 % lower rate) (Aller et al., 2001).

The success of desulfurization is also related to coal particle size because it affects bacterial ability to desulfurize coal. The particle size is determining time and velocity needed for bacteria to arrive to the particle. A reduction in total sulfur amount is more evident if particle size is smaller. It is shown that with the decrease of particle size from 4750 to 180 µm, sulfur amount is increasing. But, caused no change with smaller particle sizes (Jorjani et al., 2004). Hence, coal granulometry influence on the process success (Cardona and Márquez, 2009).

6. CONCLUSION

- Comparing sulfur removal (in %) in two investigated soils with several other investigations, it can be concluded that sulfur removal in the sample Plomin and Štalije is relatively high. So, desulfurization of soils using bacterium *Ralstonia sp.* is efficient.

- Spreading of coal contamination is in accordance with air distances of two localities where closer one, Štalije, has higher amount of initial S_t.

- Lower initial S_t, duration of bacterial treatment up to 15 days, bacterial biomass of 25 mg/mL, temperature up to 35 °C and smaller particle size seem to increase removal of sulfur in the samples.

- The environmentally sensitive elements are likely to cause environmental problems. Therefore, they are required to be removed or minimized. Due to the relatively successful desulfurization by bacterium *Ralstonia sp.*, further investigations on superhigh-organic-sulfur Raša coal should be carried out to see the effect this bacterium have on reduction of potentially toxic and trace metals in the same coal.

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