

Article

Use of Bacteria and Synthetic Zeolites in Remediation of Soil and Water Polluted with Superhigh-Organic-Sulfur Raša Coal (Raša Bay, North Adriatic, Croatia)

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Abstract: The Raša Bay (North Adriatic, Croatia) has been receiving various pollutants by inflowing streams laden with untreated municipal and coalmine effluents for decades. The locality was a regional center of coalmining (Raša coal), coal combustion, and metal processing industries for more than two centuries. As local soil and stream water were found to be contaminated with sulfur and potentially toxic trace elements (PTEs) as a consequence of weathering of Raša coal and its waste, some clean-up measures are highly required. Therefore, the aim of this study was to test the remediating potential of selected microorganisms and synthetic zeolites in the case of soil and coal-mine water, respectively, for the first time. By employing bacterial cultures of *Ralstonia* sp., we examined removal of sulfur and selected PTEs (As, Ba, Co, Cr, Cu, Ni, Pb, Rb, Se, Sr, U, V, and Zn) from soil. The removal of sulfur was up to 60%, arsenic up to 80%, while Se, Ba, and V up to 60%, and U up to 20%. By applying synthetic zeolites on water from the Raša coalmine and a local stream, the significant removal values were found for Sr (up to 99.9%) and Ba (up to 99.2%) only. Removal values were quite irregular (insignificant) in the cases of Fe, Ni, Zn, and Se, which were up to 80%, 50%, 30%, and 20%, respectively. Although promising, the results call for further research on this topic.

Keywords: coal; soil; water; bioremediation; Ralstonia sp.; sulfur; synthetic zeolite; removal

1. Introduction

Coal is a valuable resource in terms of cheap electricity production, and is also an economic source of strategically important elements (Ge, Ga, U, V, Se, rare earth elements, Y, Sc, Nb, Au, Ag, and Re) [1–3]. However, coal combustion emissions, enriched in S and potentially toxic trace elements (PTEs), such as Se, U, Cd, Hg, Pb, As, Ni, Cr, V, etc., could be hazardous for soil, water, air, and crop quality [4–7]. Coal combustion wastes (CCWs) are solid/mineral residues produced in huge quantities worldwide. If improperly disposed at unprotected landfills, their leachates, formed due to infiltrating rainwater, can contaminate local aquifers and terrestrial ecosystems with PTEs [8,9]. Soil and water degraded by the coal industry must be remediated and conserved using a strategy which needs to be achieved as cheaply and effectively as possible.

Various physical and chemical technologies of metal removal and/or stabilization from soils and sediments have been reported, each one with potential advantages and drawbacks. They include, for example, soil washing, thermal extraction, ion exchange, electrokinetic treatment, reverse osmosis,



membrane technology, evaporation recovery, solidification, plasma vitrification, etc. [10]. They are mostly very expensive, and a viable alternative could be bioremediation, which is based on the microbially mediated transformation of the metal, and its subsequent biosorption and biomineralization [11]. It has been commonly used to remove organic matter and toxic chemicals from domestic and industrial waste by means of enzymatic attacks through the activities of living micro-organisms [12]. Environmental pollutants get biodegraded due to many processes, e.g., oxidation, mineralization, transformation to toxic or nontoxic compounds, and accumulation within an organism, or they get polymerized or otherwise bound to natural materials in soils, sediments, or waters [13]. For example, Park and colleagues [10] investigated the potential bioaugmentation in sediments to improve adsorption/biosorption of Cd and Zn. A batch experiment was performed in the lake sediments augmented with *Ralstonia* sp. HM-1. A reduction of metal concentrations in the liquid phase resulted due to adsorption in sediments, with 99.7% removal efficiency for both Cd and Zn in the aqueous solution after 35 days.

Regarding coal biodegradation, by employing biological processes, coal waste gets converted to a value-added liquid product in a process known as liquefaction [14]. Although a number of microorganisms are known to be actively involved in it, and for some, the extracellular enzymes involved are also known, the underlying mechanism(s) employed in the process of coal biodegradation are not yet fully established, partly due to the highly complex structure of coals [14]. It is a naturally complex process driven by an array of extracellular enzymes in the presence of various chelators and supporting enzymes released by different microorganisms that coinhabit the coal environment [15]. For example, bioremediation was successfully developed and applied on Indian and Indonesian coals using mixed bacterial consortium, which effectively removed more than 80% of Ni, Zn, Cd, Cu, and Cr, and 45% of Pb [16]. Also, the same research team has carried out desulfurization of coal using microorganisms Ralstonia sp. and Pseudoxanthomonas sp. [17], and found out the highest positive correlation between initial total S (2.2 Wt% dry ash free basis) and removal percentage (up to 45%) in the case of Indian Vastan lignite treated with Ralstonia sp. Ralstonia sp. are gram-negative, non-fermentative, rod-shaped bacteria ubiquitously present in soil and water [18]. They exhibit many advantages for the cleaning treatments due to their large range of environmental conditions, biodegradative abilities, and large metabolic diversity [19]. It was suggested [18] that *Ralstonia* sp. strain OR214 was tolerant to high concentrations of PTEs such as Cd, Co, Ni, and U.

Surface water pollution caused by coalmine discharges laden with toxic pollutants, especially PTEs, is a widespread problem and, occasionally, national standards for wastewater discharges from mines are violated [20]. Since PTEs are not biodegradable, they tend to accumulate in aquatic organisms, causing various diseases and disorders. Therefore, numerous treatment technologies have been developed to remove PTEs from wastewater, e.g., chemical precipitation, coagulation, ultra-filtration, biological systems, electrolytic processes, reverse osmosis, oxidation with ozone/hydrogen peroxide, membrane filtration, and ion exchange [21]. Due to their high cost and disposal problems, many of these conventional methods have not been widely applied on a large scale. Adsorption has been found to be superior compared to other techniques in terms of initial cost, flexibility and simplicity of design, ease of operation, and insensitivity to toxic pollutants [21]. Zeolites, natural as well as synthetic, represent low-cost materials with high adsorption selectivity for cationic contaminants [21–24]. Studies conducted on model solutions showed that synthetic zeolites, compared to natural ones, had higher cation-exchange capability, and consequently better PTE sorption performances [25].

The Raša Bay (North Adriatic, Croatia; Figure 1) has been receiving various pollutants by inflowing streams laden with untreated municipal and coalmine effluents for decades [20]. Locally mined superhigh-organic-sulfur (SHOS) Raša coal is a unique variety compared to other coal types worldwide based on its anomalously high levels of S, Se, and U [5]. The whole local area (about 600 km²) has experienced damaging environmental impacts of SHOS Raša coal due to mining, preparation, combustion, waste storage, and transport in the past [26]. Soil locations downwind from a local coal-fired power plant are severely polluted with S (up to 4.00%), Se (up to 6.80 mg/kg), Cd (up to

4.70 mg/kg), and organic contaminants, such as PAHs, up to 13,500 ng/g [4,27]. In particular, Selenium and selected PTEs were found to be increased in soil, sediment, surface water, locally grown vegetables, and local birds [5,28]. A local CCW, located in a Štrmac village (Figure 1, S4 and S8 locations), was found to have decreased Se values compared to relevant previous studies [29]. This indicates leaking problems, as the study area belongs to vulnerable karstic environment which promotes Se mobility due to high Eh/pH conditions [5].



Figure 1. Map of the study area. (a) Red dot—study area (east side of the Istrian Peninsula). (b) Water sampling points: CME—coal-mine effluent (water samples no. 1 and 2, inside of a Raša coal-mine in Krapan village), water samples no. 3, 4, and 5 taken from the Krapan stream. (c) Soil sampling sites no. S2–S14. Š.—a former Štalije coal-separation unit, R.—the Raša town, S9—Krapan village.

The objectives of this study were as follows: (1) To apply bioremediation tests, by using *Ralstonia* sp. on SHOS Raša coal, as well as few selected soil samples polluted with SHOS coal and CCW, geochemically characterized in previous papers [4,5,20], and (2) to examine the ability of synthetic zeolites to remove PTEs from Raša coalmine water and stream water affected by coal leachates and nontreated municipal wastewater on laboratory scale.

2. Materials and Methods

2.1. Sampling and Sample Preparation

Soil and SHOS Raša coal sampling campaigns (Figure 1) were conducted on several occasions during the period 2013–2015 [4,20,28]. Soil samples were collected at: S2, a former small-scale coal-fired power plant Vlaška; S4, and S8, CCW locality in Štrmac village; S6, sampled close to a beach in the Trget village; S9, a former coal-mining town Krapan; S14, a former rail line (red-colored line in Figure 1), between the Raša town (R.) and a coal-separation unit Štalije (Š.); and S10, unpolluted soil [30] taken from a site 10–15 km NE from the study area. A summary of soil samples' (2 kg per sample) physicochemical properties (pH, LOI—loss on ignition, CEC—cation exchange capacity, and CaCO₃, acquired and described in previous papers [4,20,28]), along the levels of sulfur, PTEs in soil, and SHOS Raša coal, are shown in Table 1. Nonfiltered water samples (300 mL per sample) were collected from an old mining town Krapan and a city of Raša (Figure 1) in early May 2019. Raša coalmine water (n = 2) was sampled from the floor inside of an underground corridor in Krapan. Then, surface water (n = 2) was taken from a Krapan stream, which flows roughly parallel to the rail line (red line in Figure 1)

and receives coa-mine water. The same stream was sampled (n = 1) downstream in the city of Raša, immediately after the point where municipal wastewater effluent (MWE) was being discharged into the stream. Samples were collected from the surface at a maximum depth of 10 cm in acid-cleansed plastic bottles. Zeolite treatments and PTEs measurements were carried out the following day.

Table 1. Basic physicochemical characteristics of soil (CaCO₃, and loss on ignition (LOI) in %; cation exchange capacity (CEC) in mEq/100), and values of S (%) and PTEs (mg/kg) in soil and Raša coal (na—not analyzed, DL—detection limit). Soil sample sites shown in Figure 1.

	Site	pН	CaCO ₃	LOI	CEC	S	Se	V	U	Sr	Cr	Cu	Pb	Zn
S2	Vlaška	7.6	56	15	23	2.1	10	318	11	655	73	13	75	11
S4	CCW na na na na		na	0.7	3.3	118	5.7	285	89	1800	200	6580		
S6	Trget	7.6	36	19	6	0.8	<dl< td=""><td>52</td><td>5.0</td><td>169</td><td>45</td><td>625</td><td>210</td><td>936</td></dl<>	52	5.0	169	45	625	210	936
S8	CCW	7.2	39	25	22	2.5	1.8	72	1.8	277	52	111	69	334
S9	Krapan	7.1	12	19	12	0.9	<dl< td=""><td>560</td><td>0.2</td><td>74</td><td>1860</td><td>1847</td><td>72</td><td>953</td></dl<>	560	0.2	74	1860	1847	72	953
S14	Rail	6.9	3	83	6	6.9	27	264	8.2	356	100	190	46	863
S10	Unpolluted	6.8	0	15	20	0.2	1.5	229	4.3	82	135	41	56	169
С	Coal	na	na	na	na	10.6	23	80	14	290	27	6	3	32

2.2. Bioremediation Tests

Soil and SHOS Raša coal samples were treated with a known bacterial species, *Ralstonia* sp., which was grown, separated, and mass cultivated under the optimum conditions, temperature of 35 °C, and growth pH of 7, in the Bioremediation lab, Department of Botany, Banaras Hindu University, and identified in the Institute of Microbial Technology (IMTECH), Chandigarh (India) on the basis of biochemical characteristics [16]. The exponential phase bacterial cells (1000 mg), representing the growth phase of bacteria where bacterial cells divide as fast as possible by intake of nutrients from GPY (i.e., glucose, peptone, and yeast extract) culture media, was obtained through the centrifugation at a rate of 10,000 rpm for 10 min, then washed two times with double-distilled water to remove any sort of contamination and extracted of growth media. The resultant bacterial biomass was mixed into 100 mL of 5% (w/v) solution of sodium alginate (NaC₆ H_7O_6), prepared in the GPY medium. The mixture was pumped dropwise into the 0.2 M CaCl₂ solution, which served as a gelling agent to provide more stable beads (preferably in the laminar flow cabinet, to avoid contamination). The formed beads were harvested and resuspended in a 100 mL growth medium (GPY) in a 250 mL cotton plugged culture flask. Soil samples were initially sterilized under ultraviolet rays for up to 45 min in the laminar flow cabinet prior to the treatment. Then, 5.0 g of each sterilized sample was taken in a conical flask containing 75 mL of sterilized distilled water, and 100 beads (equivalents to 1000 mg dry weight of bacteria) were added in each flask. It was calculated that from 1 mL solution of biomass and sodium alginate, 25 beads were formed. For the creation of 100 beads, 4 mL of solution was required. Likewise, it was calculated that 1 mL of the solution of bacterial biomass in sodium alginate contained 250 mg of dry weight of bacteria. Therefore, 4 mL of the solution, which was required for 100 beads, had to contain 1000 mg of dry weight of bacteria. The treatment was carried out for 18 days, and analysis of sulfur content was performed after 6, 12, and 18 days, whereas analysis of PTEs was carried out on 12-day treatment samples. Bioremediation of SHOS Raša coal sample was also carried out for 7 days, and residual sulfur content was analyzed on 3-, 5-, and 7-day treatment samples.

2.3. Adsorption Experiment

Analysis and experiment were done on original, unfiltered samples, excluding sample no. 3, which was filtered due to high amount of suspended material. Water samples were prepared by 10-fold dilution, followed by the addition of 2% (v/v) HNO₃ s.p. and In (1 μ g/L) as an internal standard. Aliquots of each sample were used for the adsorption experiment. Two types of synthetic zeolites were used as follows: (1) (Z) Purmol 4ST, composed of zeolite (90%) and water (10%); white color, pH 9–11.5, and (2) (A) 3A-50% (activated zeolite), i.e., synthetic sodium potassium zeolite; pH 10.1–11.4.

The adsorption experiment was carried out as follows: Aliquots (20 mL) of water samples were placed into plastic bottles containing accurately weighed amounts of the sorbents, i.e., (*Z*) Purmol 4ST (m = 1.0 g), and (A) 3A-50% (m = 1.0 g). The prepared suspensions were shaken using a mechanical shaker at 320 rpm. Following a contact time of 120 min, 5 mL of the suspension was taken by a syringe from each bottle and filtered through a 0.45-µm filter. Prior to analysis, the obtained solutions were diluted 10 times, acidified with 2% (v/v) HNO₃ (65%, suprapur, Fluka, Steinheim, Switzerland), and In (1 µg/L) was added as an internal standard.

2.4. Analysis of Sulfur and PTEs

Total sulfur contents (%) in original and bioremediated soil (fraction < 0.063 mm), and reference material ISE 979 (Rendzina soil from Wepal, the Netherlands) were determined using Eschka's mixture according to the standard test method [31]. Accuracy and precision were within 10% between analyzed and certified values.

The multi-element analysis of prepared nontreated soil samples was carried out with X-ray fluorescence (XRF). Prior to analysis, reference standard soils, MESS3 and NIST, were used for the data calibration [32]. All samples and standards were dried in an oven for two days at 60 °C, ground, and sieved through 200 ASTM sieve plate. The material (4 g) was mixed with 1 g of boric acid powder, and the mixture was homogenized. No trace of boric acid clump was left to avoid background scattering peaks. Samples were placed in press pellet using a KBr press pellet machine. Analysis was conducted with X-ray beam until the measurement was recorded by an automated detector inside the XRF (S8 TIGER, Bruker, Mannheim, Germany) analyzer.

Prior to high-resolution inductively coupled plasma mass spectrometry (HR-ICP-MS) analysis of bioremediated soil samples, subsamples (0.05 g) were subjected to total digestion in the microwave oven (Multiwave ECO, Anton Paar, Graz, Austria) in two-step procedure consisting of digestion with a mixture of 4 mL nitric acid (HNO₃)—1 mL hydrochloric acid (HCl)—1 mL hydrofluoric acid (HF), followed by addition of 6 mL of boric acid (H₃BO₃). Prior to analysis, sample digests were diluted 10-fold, acidified with 2% (v/v) HNO₃ (65%, supra pur, Fluka, Steinheim, Switzerland), and In was added (1 μ g/L) as an internal standard. Details of the sample preparation protocols, description of instrument conditions, and measurement parameters are reported elsewhere [33]. Analysis was conducted with an Element 2 instrument (Thermo, Bremen, Germany). Standards for multi-element analysis were prepared by appropriate dilution of a multi-elemental reference standard (Analytika, Prague, Czech Republic) containing Al, As, Ba, Be, Bi, Cd, Co, Cr, Cs, Cu, Fe, Li, Mn, Ni, Pb, Se, Sr, Ti, Tl, V, and Zn, in which single-element standard solutions of Rb, Sb, Sn, and U (Analytika, Prague, Czech Republic) were added. All samples were analyzed for total concentration of following elements: As, Ba, Co, Cr, Cu, Ni, Pb, Rb, Se, Sr, U, V, and Zn. Quality control was performed by simultaneous analysis of the blank and the certified reference material for soil (NCS DC 73302, also known as GBW 07410, China National Analysis Center for Iron and Steel, Beijing, China). Good agreement (±10%) between analyzed and certified concentrations was obtained for all measured elements.

Multi-elemental analysis of prepared original and zeolite-treated solutions was performed by HR-ICP-MS using an Element 2 instrument (Thermo, Bremen, Germany). External calibration was used for the quantification. All samples were analyzed for total concentration of following elements: As, Ba, Cd, Cr, Cu, Fe, Mo, Ni, Pb, Se, Sr, U, V, and Zn. Typical instrument conditions and measurement parameters used throughout the work are reported elsewhere [33].

2.5. Data Analysis

Data analysis was conducted with free PAST software [34]. It included calculations of basic statistical parameters and Kendall's Tau correlation coefficients. Level of significance was 0.05.

3. Results and Discussion

3.1. Bioremediation of SHOS Raša Coal and Polluted Soil

3.1.1. Geochemical Characterization of Coal and Soil

Table 1 shows basic physicochemical characteristics of soil from the Raša Bay area, along a background (unpolluted) soil sample taken some 10–15 km away from the study area (Figure 1), and values of PTEs in soil and Raša coal.

Evidently, Raša soil is polluted with all analyzed PTEs and S compared to unpolluted soil collected far from the study area [4,5,20,28]. Briefly, S, Se, V, and U were elements indicative for Raša coal weathering, both from underground deposits and surface coal waste piles abandoned across the study area [1,5,26]. Strontium is typical for marine and karst environments, while Cr, Cu, Pb, and Zn commonly result from metal processing industries [20]. Samples S4 and S9 were collected from the Strmac village, where various coalmining and foundry factories, which closed in the late 1950s, were previously active. Anomalously high levels of Cu, Pb, and Zn in the sample S4, collected above the CCW site (Figure 1), resulted from foundry waste, mixed with CCW. All the analyzed variables (Table 1) were used for the correlation analysis to see their mutual relations. Table 2 shows that S was positively correlated only with Se, but not significantly, while its relations with U and V were surprisingly very weak. Positive statistically significant relations were as follows: Se-U, Se-Sr, U-Sr, V-Cr, and Pb-Zn. The first three correlations indicate on weathering of Raša coal particles (Se and U), concomitant with weathering of karst bedrock (Sr), while V, Cr, Pb, and Zn are characteristic for metal processing activities [20]. Expectedly, LOI was positively correlated with S, i.e., coal particles, not significantly though. It is intertesting to note that two samples, one taken from a vicinity of the Trget beach, had lost all their Se (Table 1), presumably due to leaching processes [35].

	pН	CaCO ₃	LOI	CEC	S	Se	V	U	Sr	Cr	Cu	Pb	Zn
pH		0.07	0.10	0.27	0.19	0.58	0.43	0.43	0.79	0.19	0.43	0.07	0.43
CaCO ₃	0.73		0.19	0.07	0.62	0.79	0.99	0.62	0.32	0.62	0.14	0.32	0.14
LOI	-0.66	-0.52		0.27	0.19	0.41	0.79	0.79	0.79	0.79	0.79	0.07	0.79
CEC	0.44	0.73	-0.44		0.79	0.58	0.43	0.79	0.43	0.79	0.19	0.79	0.19
S	-0.52	-0.20	0.52	0.10		0.24	0.85	0.85	0.57	0.85	0.34	0.03	0.18
Se	-0.22	0.10	0.33	0.22	0.41		0.43	0.05	0.01	0.43	0.24	0.24	0.43
V	-0.31	0	-0.10	0.31	0.06	0.27		0.57	0.34	0.03	0.85	0.34	0.85
U	0.31	0.20	-0.10	0.10	0.06	<u>0.69</u>	0.20		0.01	0.85	0.18	0.85	0.34
Sr	0.10	0.40	0.10	0.31	0.20	<u>0.82</u>	0.33	<u>0.86</u>		0.85	0.09	0.57	0.18
Cr	-0.52	-0.20	0.10	0.10	0.06	0.27	<u>0.73</u>	-0.06	0.06		0.34	0.34	0.57
Cu	-0.31	-0.60	0.10	-0.52	-0.33	-0.41	0.06	-0.46	-0.60	0.33		0.85	0.01
Pb	0.73	0.40	-0.73	0.10	<u>-0.73</u>	-0.41	-0.33	-0.06	-0.20	-0.33	0.06		0.57
Zn	-0.31	-0.60	0.10	-0.52	-0.46	-0.27	-0.06	-0.33	-0.46	0.20	<u>0.86</u>	0.20	

Table 2. Kendall's Tau correlation coefficients (below the diagonal) of the variables shown in Table 1 (bold italic underlined significant at p < 0.05; p values displayed above the diagonal).

3.1.2. Desulfurization of Coal and Soil

Figure 2 presents the results of desulfurization conducted on SHOS Raša coal. It shows that maximum removal of its sulfur, which was almost entirely in organic form [5,26], was up to 5% at best, i.e., not significant. However, it shows a general trend of decreased S concentrations in coal with time. Raša coal desulfurization was far less than S removal (9%) found in the case of Assam coal, which had 5–6% S, mainly in organic form, but also in pyritic form to some extent [36]. No dissolution of organic + sulfate S was found, as Thiobacillus ferrooxidans was not able to remove organically bound S [36]. Sulfur removal from Assam coal was only 9% compared to 91% from lignite, which had high content of pyritic S. It was also found that poor removal of S from Assam coal was due to extensive precipitation

of jarosites, which was reflected in the maximum increase in the volatile matter in microbially treated Assam coal [36].



Figure 2. Sulfur concentrations (%) in original (0 day), and bioremediated (3-, 5-, and 7-day treatment periods) samples of SHOS Raša coal.

Microbial coal desulfurization, specifically the inorganic S, involves a complex combination of nonbiological and microbiologically catalyzed oxidations of sulfide minerals into sulfates, which are soluble in water. Herewith, desulfurized coal gets separated from liquid phase and washed with water [36]. The efficiency of bacterial bioremediation depends a lot on the type of sulfur species present in coal [37]. The sulfur-containing compounds in coal can be present as both aliphatic sulfur-containing chains (mercaptans, aliphatic sulfides, thiophenes) and heteroatoms in aromatic rings [38,39]. In high-sulfur coals, the relative proportion of aliphatic structures to total organic sulfur appears to be in the range of 30–50% [38]. Either in organic or pyritic form, 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 [39,40]. High molecular weight compounds, such as aromatic ones, are usually more stable, and therefore heavier to break by bacteria [41,42]. Measurements made on Indonesian and Indian coals by means of *Ralstonia* sp. showed sulfur removal from 6% to 68% [17]. Also, investigations conducted on a same Indian coal sample aimed at comparison of desulfurization capacity of two bacteria, Pseudoxanthomonas sp. and Ralstonia sp., showed a better performance of the previous one [17,43]. Results from other investigations of coal remediation using other bacterial species showed sulfur removal values of 27%, 50%, and 31–51% [42,44,45].

The desulfurizing potential of *Ralstonia* sp. tested on soil polluted with SHOS Raša coal and CCW is presented in Figure 3. Values of removal of S (%) in samples S2, S4, S6, S8, S9, and S10 were up to 50, 30, 40, 60, and 20, respectively. Similarly to desulfurization in SHOS Raša coal, desulfurization of soil increased with time. The highest removal was recorded in a sample collected from the CCW site (Štrmac locality), hosting a huge quantity of SHOS Raša coal combustion byproducts, as well as waste from a former foundry factory (closed in the late 1950s). The lowest removal was exhibited by the unpolluted soil sample collected away from the Raša locality (Figure 1). Hereby, the calculated Kendall's Tau correlation index between S removal values (%) and initial total S levels in soil (%) was 0.84. A similar finding was reported by [17], which determined a positive correlation between total S (2.2 Wt% dry ash free basis) and removal percentage levels (up to 45%).



Figure 3. Soil total sulfur (%) in original (0 day), and bioremediated (6-, 12-, and 18-day treatment periods) samples. S10—unpolluted soil; S2, 6, and 9—soil dominantly polluted with SHOS Raša coal particles; S4, and 8—soil dominantly polluted with CCW particles.

3.1.3. Demineralization of Soil

Table 3 presents comparison of levels of PTEs in untreated and bioremediated soil samples with calculated removal values (%). Similar to soil desulfurization, lowest PTE removals were found for the unpolluted soil sample S10. Anomalously elevated Cu and Zn levels in the sample S4, both initial and bioremediated, are related to previously active practice of dumping foundry waste enriched in heavy metals [20]. Compared to initial Cu value, the bioremediated Cu was almost two-fold, the cause of which remains unclear, and it should be examined by future detailed microbiological studies. Almost all removal values in the case of two polluted soil samples (S4 and S14) were higher compared to the ones calculated for the unpolluted (S10) soil sample. The highest PTE removal values, except for Se and U, were observed for the sample S14. It was collected from a site of the former coal-train rail track Raša-Štalije (R.-Š., red-colored line in Figure 1). The Štalije location was a coal separation unit in the past. At the site S14, normally red-colored terra rossa soil is almost black due to dispersed coal particles fallen from coal-train coaches in the past [20]. The highest removal values in the case of the sample S10 are listed in descending order as follows: Rb > Ni = Zn > As = Ba = Cr = Cu = Sr = V >Pb > Co = U. The highest removal values in the case of the sample S4 were in following descending order: Ba = Se > V > Cr = Ni = Rb = Zn > As = Co = Pb > U. In the case of the sample S14, descending order of removal levels was following: As = > Ba = Co = Cu = Ni = Rb = Sr = V = Zn > Cr = Pb > U. Herewith, the lowest removal values were exhibited by U and Pb.

Similar to desulfurization, correlation indices between PTE removal levels (%) and initial total PTE levels (mg/kg) for soil samples S10, S4, and S14 were 0.60, 0.14, and 0.13, respectively. Herewith, soil desulfurization and demineralization trends had similar patterns, and they were also similar to previously found relevant relations for coal [16,17]. Evidently, bioremediation experiment carried out in this study was fairly successful. Moreover, its performance is comparable with coal PTE removals found by an earlier study [46], which were as follows: As (53%), Cu (39%), Co (42%), Cr (13%), Zn (31%), Ni (34%), and Pb (65%). The study [10] reported the potential of *Ralstonia* sp. HM-1, one of the bacteria resistant to potentially toxic metals, to improve adsorption onto lake sediments and biostabilization of Cd and Zn. Batch experiments were conducted using the spike of the synthetic Cd and Zn stock solution to investigate the effect of both the indigenous microorganism in sediment

and the inoculation of *Ralstonia* sp. HM-1 in the bottle containing sediment and surface lake water. The reduction of the exchangeable fraction and the increase of bound organics and sulfide fractions were observed with the addition of *Ralstonia* sp. HM-1, showing its role in the prevention of metal elution from the sediment to water phase [10].

		S10			S 4		S14				
	Initial	Final	Removal (%)	Initial	Final	Removal (%)	Initial	Final	Removal (%)		
As	30.8	23.3	20	8.82	5.55	30	46.5	5.02	80		
Ba	227	174	20	392	135	60	147	48.8	60		
Co	19.3	18.2	0	9.05	6.22	30	9.30	3.05	60		
Cr	135	106	20	89.7	47.7	40	100	42.6	50		
Cu	41.4	31.3	20	1800	3000	-	190	71.7	60		
Ni	123	80.6	30	52.3	26.5	40	60.3	19.5	60		
Pb	56.4	48.9	10	200	132	30	46.3	20.3	50		
Rb	122	67.7	40	30.4	18.0	40	23.7	9.40	60		
Se	1.50	1.35	10	3.31	1.00	60	27.5	27.0	0		
Sr	82.2	63.0	20	285	328	-	356	127	60		
U	4.30	4.18	0	5.76	4.23	20	8.20	7.07	10		
V	229	167	20	118	58.2	50	264	93.6	60		
Zn	169	112	30	6580	3320	40	863	331	60		

Table 3. Levels of PTEs (mg/kg) in nontreated (initial), and bioremediated (final, 12-day treatment) soil samples, with calculated removal values (na—not analyzed).

S10—unpolluted soil, S4—soil dominantly polluted with CCW ash, S14—soil dominantly polluted with Raša coal particles.

3.2. Zeolite Adsorptive Removal of PTEs from Coalmine and Stream Water Samples

Initial total levels of Se, U, V, and Mo in water samples, elements highly enriched in SHOS coals [5], are presented in Figure 4. Their close association, characteristic for SHOS Raša coal, was evidenced by statistically significant (p < 0.05), highly positive correlation coefficients (>0.99), reported earlier [5]. Selenium replaces S in organic complexes, and Se-bearing coals are exclusively high-S coals [47]. World stream water values [48] of Se, U, V, Mo, Cd, Ni, Sr, and Ba are as follows (μ g/L): 0.2, 0.04, 0.9, 0.5, 0.02, 0.3, 70, and 20, respectively. Compared to them, Se was increased 35-45 times, U 45-55 times, V 3–5 times, Mo 25–35 times, Cd 6–9 times, Ni 3–6 times, Sr 10–13 times, and Ba 1.3–1.7 times. All the other elements were comparable to world stream water data [48]. By considering the downstream trend of sampling points, i.e., no. 1–5, only Pb, Fe, Cu, Zn, and As had increasing concentrations, while values of other PTEs were rather similar regardless of the sampling sites, shown in Figure 5 for Se, Mo, U, and V concentrations. Kendall's Tau correlation coefficients of the analyzed PTEs are shown in Table 4. They were predominantly positive, and several of them were significant. It should be noted that Se was not significantly correlated with either of analyzed PTEs, and it was even negatively correlated with few of them (Pb, Cu, Zn, and Ba). Selenium is known as an essential toxin due to its narrow range between dietary essentiality and toxicity for lifeforms. According to the study [35], waterborne Se levels of $2-5 \mu g/L$ pose concern to the aquatic life. Therefore, it is of interest to monitor Se levels in coalmine water and surface streams fed by it. As can be noticed from Figure 4, Se levels are comparable with previous studies [5,20,28], and are close to the Croatian regulatory limit value of $10 \mu g/L$. In comparison to the world data, these values were much above the average total Se measured $(\mu g/L)$ in wastewater from Spain (0.13), Belgium (0.35), Israel (0.44), Germany (0.12), Netherlands (0.12), and New York (<0.2) (references in [28]).



Figure 4. Initial Se, Mo, U, and V total concentrations (μ g/L) in water samples collected as follows: 1, 2: coalmine water; 3, 4: Krapan stream, downstream of coalmine; and further downstream, sample no. 5: Krapan stream, downstream of municipal wastewater effluent.



Figure 5. Water (1, 2: coalmine water; 3, 4: Krapan stream, downstream of coalmine; 5: Krapan stream, downstream of municipal wastewater effluent) Ba and Sr total values (vertical axis in log scale). 1—initial value, 2—value following the zeolite Purmol 4ST (Z) treatment, and 3—value following the activated zeolite 3A-50% (A) treatment.

Table 4. Kendall's Tau correlation coefficients (below the diagonal) of the variables measured in coalmine and surface water samples (bold italic underlined significant at p < 0.05; p values displayed above the diagonal).

	Cd	Pb	Cr	Fe	Ni	Cu	Zn	Sr	Ba	As	Se	Мо	U	V
Cd		0.79	0.07	0.79	0.19	0.79	0.43	0.07	0.43	0.79	0.27	0.02	0.07	0.07
Pb	-0.10		0.62	0.05	0.32	0.01	0.14	0.62	0.62	0.32	0.79	0.99	0.62	0.62
Cr	0.73	0.20		0.32	0.05	0.62	0.14	0.14	0.14	0.32	0.43	0.05	0.14	0.01
Fe	0.10	<u>0.80</u>	0.40		0.62	0.05	0.32	0.99	0.32	0.14	0.79	0.62	0.99	0.32
Ni	0.52	0.40	<u>0.80</u>	0.20		0.32	0.05	0.05	0.32	0.62	0.79	0.14	0.05	0.05
Cu	-0.10	<u>0.99</u>	0.20	<u>0.80</u>	0.40		0.14	0.62	0.62	0.32	0.79	0.99	0.62	0.62
Zn	0.31	0.60	0.60	0.40	<u>0.80</u>	0.60		0.14	0.14	0.32	0.79	0.32	0.14	0.14
Sr	0.73	0.20	0.60	0	<u>0.80</u>	0.20	0.60		0.62	0.99	0.79	0.05	0.01	0.14
Ba	0.31	0.20	0.60	0.40	0.40	0.20	0.60	0.20		0.05	0.79	0.32	0.62	0.14
As	0.10	0.40	0.40	0.60	0.20	0.40	0.40	0	<u>0.80</u>		0.79	0.62	0.99	0.32
Se	0.44	-0.10	0.31	0.10	0.10	-0.10	-0.10	0.10	-0.10	0.10		0.43	0.79	0.43
Mo	<u>0.94</u>	0	<u>0.80</u>	0.20	0.60	0	0.40	<u>0.80</u>	0.40	0.20	0.31		0.05	0.05
U	0.73	0.20	0.60	0	<u>0.80</u>	0.20	0.60	<u>0.99</u>	0.20	0	0.10	<u>0.80</u>		0.14
V	0.73	0.20	<u>0.99</u>	0.40	0.80	0.20	0.60	0.60	0.60	0.40	0.31	0.80	0.60	

The results of the zeolite adsorptive removal of PTEs from coalmine and surface water samples are presented in Table 5, and Figure 5.

Table 5. Water (Loc. (location)—1, 2: coalmine water; 3, 4: Krapan stream, downstream of coalmine; 5: Krapan stream, downstream of municipal wastewater effluent) PTE total values (μg/L). Tr. (treatment): 1—inital value, 2—zeolite Purmol 4ST (Z), and 3—activated zeolite 3A-50% (A).

Loc.	Tr.	Мо	Cd	Pb	U	V	Cr	Fe	Ni	Cu	Zn	As	Se	Ba	Sr
1	1	16.7	0.18	0.08	2.20	4.10	0.89	10.6	1.40	0.49	6.81	0.35	8.90	27.6	908
	2	18.4	0.16	0.07	2.31	5.30	10.1	15.9	1.43	2.26	4.64	0.84	11.4	1.57	27
	3	32.4	0.26	0.21	3.11	14.5	2.93	46.0	0.78	1.14	7.55	2.30	14.9	0.39	0.24
-	1	12.8	0.14	0.09	1.84	3.00	0.73	25.8	0.98	0.62	5.01	0.38	9.10	26.1	721
2	2	14.0	0.12	0.06	1.91	4.20	10.1	19.6	0.89	0.73	3.46	0.99	8.90	0.33	3.00
	3	13.9	0.12	0.14	1.37	7.00	1.23	5.00	0.41	0.50	3.57	1.77	9.30	0.21	0.54
	1	12.2	0.12	0.19	1.85	2.60	0.67	12.0	1.00	1.02	6.65	0.13	7.00	25.7	732
3	2	13.3	0.13	0.24	2.12	8.50	9.50	168	1.24	4.60	11.3	1.21	9.00	0.95	1.21
	3	13.1	0.12	0.20	1.76	10.4	1.20	29.1	0.65	1.97	6.85	1.75	9.50	0.29	1.29
-	1	14.4	0.15	0.34	1.94	4.60	0.90	81.3	1.69	3.24	14.5	0.62	8.90	33.6	798
4	2	15.1	0.14	0.36	1.95	5.20	9.92	33.6	2.63	3.23	13.8	1.36	9.10	0.55	3.84
	3	14.9	0.14	0.17	1.65	8.20	1.32	20.7	1.60	3.24	17.1	1.77	7.10	0.50	2.94
	1	13.7	0.14	0.27	1.87	3.90	0.78	26.6	1.22	2.31	13.0	0.65	7.70	33.7	786
5	2	15.0	0.14	0.20	1.96	4.90	9.80	34.0	2.63	2.97	10.5	1.09	8.20	1.02	9.34
2	3	15.0	0.14	0.89	1.72	8.10	1.39	32.2	1.29	2.50	10.9	1.82	7.30	1.65	18.6

The best removal efficiencies were found for Ba and Sr. The results presented in Figure 5 demonstrate the decrease of Ba and Sr for the both zeolite treatments in all water samples except of the sample no. 5. In the case of Ba, removal values (%) of the zeolite Purmol 4ST (Z), and the activated zeolite 3A-50% (A) for the samples 1–5 were as follows, respectively: 94.3 and 98.6; 98.7 and 99.2; 96.3 and 98.9; 98.4 and 98.5; and 97.0 and 95.1. Excluding sample no. 5, the A zeolites exhibited better performances compared to the Z ones. In the case of Sr, removal values (%) of the Z, and A for the samples 1–5 were as follows, respectively: 97.0 and 99.9; 99.6 and 99.9; 99.8 and 99.8; 99.5 and 99.6; and 98.8 and 97.6. Again, excluding sample no. 5, the A zeolites exhibited better performances compared to the Z ones. It seems that the sample no. 5, impacted by both coalmine and MWE effluents, was more challenging in terms of purification with the applied zeolites compared to the other four water samples. Possibly, it could be ascribed to increased levels of Pb, Fe, Cu, Zn, and As in the sample no. 5, as lower removal efficiencies have been referred [25] in conditions of increased PTE concentrations. However, at very low PTE concentrations, the effects of zeolites likely become negligible, as was probably the case with Pb levels in this study. By converting 2.5 mmol Pb/L, reported in [25], it is about 518 mg/L, which is much higher than Pb data in this study (Table 5). Commonly, tests of water treatment with zeolites operate with PTE concentrations in the range $100x \ \mu g/L$ to $100x \ m g/L$. It was not possible to compare the results of this study (Table 5) with literature values. To the best of our knowledge, there are no published similar studies focused on karst coalmine effluents treated with zeolites. Herewith, the removal effects of the applied zeolites were observed in the case of Sr and Ba only, which can be ascribed to their highest concentrations in the initial samples compared to the rest of analyzed PTEs. Also, there is no single process capable of adequate water treatment, mainly due to the complex nature of the effluents [21]. A combination of different processes is commonly necessary to achieve the desired water quality in the most economical way. The paper [21] reviewed adsorption capacities of various industrial wastes in terms of heavy metal removal. It demonstrated that adsorption capacities of the adsorbents varied depending on the characteristics of the adsorbents, the extent of chemical modification, and the concentration of adsorbates. Generally, percent adsorption increased with increased adsorbent dose, contact time, and agitation speed. It should be noted that for every investigated element, there was a favorable pH range in which maximum adsorption occurred [21]. Differently to Ba and Sr, the results

are specific to few samples, as in several situations, post-treatment concentrations were comparable, or even higher, than the initial ones. In conclusion, by considering a +/-10% variation of the values in Table 5, typical for many analytical methods, the effect of zeolites was significant for Ba and Sr only.

4. Conclusions

The present study applied bacterial cultures of *Ralstonia* sp. on SHOS Raša coal and soil polluted with coal and coal-combustion waste. The removal of organically bound sulfur from coal was negligible (up to 5%), while soil desulfurization was up to 60%. Values of removal of PTEs from soil were as follows: Up to 80% for As, and Mo, up to 60% for Se, Ba, and V, and up to 20% for U. By applying synthetic zeolites on SHOS Raša coalmine water and municipal wastewater, the significant removal was found for Sr (99.9%) and Ba (99.2%) only. The activated zeolites (3A-50%) were slightly more efficient sorbents for PTEs compared to the Purmol 4ST zeolites. The overall conclusion is as follows: The examined microorganisms could be used for soil cleanup in terms of sulfur and the abovementioned PTEs, which is a cost-effective alternative compared to various chemical and physical methods. Regarding PTE removal from SHOS Raša coalmine water, synthetic zeolites proved excellent sorbents for Ba and Sr, while Se, which is most important among PTEs at the study locality, should be examined by future multidisciplinary research.

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