Effects of microscale particles in red mud amended artificial soils on bioaccumulation of elements in *E. fetida*

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Abstract

Red mud (RM) contains large quantities of microscale particles < 1 μm and high concentrations of potentially toxic elements. In this research, we have used two types of RM of similar chemical properties but containing different quantities of micro-particles, to test whether their size plays a role in the uptake of chemical elements by earthworm *Eisenia fetida*. Earthworms were exposed for seven days to artificial soils (prepared in the laboratory following a protocol) amended with increasing quantities of RM. Mortality of 86% occurred when earthworms were exposed to amended soil containing 46% of particles below 1 μm. Surprisingly, tissue analyses have shown decreased concentrations of metals instead of the expected toxic effect. SEM analysis revealed that micro-particles strongly adhere to the earthworm epidermis putting them under the large stress. Micro-particles in RM clog their

minute dermal pores of 90 nm to 735 nm in diameter, which size depends on whether the earthworm's body is contracted or stretched. Strong adhesion of micro-particles to earthworms' epidermis and blockage of their microsize pores prevented normal dermal respiration and absorption of chemical elements through their epithelium resulting in a decrease of most measured metals, especially essential elements potassium, calcium and iron, followed by the lethal outcomes.

Keywords: red mud, microscale particles, *Eisenia fetida*, dorsal pores, dermal pores, elements uptake, potential deficiency of K, Ca and Fe, artificial soil test

1. Introduction

Red mud (RM) is a by-product of bauxite refinement using the Bayer process in order to produce alumina. Depending on the quality of the bauxite, 1-2.5 tons of RM are generated per ton of alumina produced (Paramguru et al., 2004). The Bayer process is more efficient when the bauxite ore is reduced to a very fine particle size prior to reaction (Paramguru et al., 2004), and this is reflected in the RM which usually contains a high amount of particles below 1 μm.

RM consists of Fe, Al, Ti, combined soda and silica as the bulk constituents (Paramguru et al., 2004) and may contain elevated concentrations of potentially toxic metals (e.g. V, Cr, Ni, Cu, Zn, Pb) (Kutle et al., 2004), metalloids (e.g. As) (Obhođaš et al., 2012) and radionuclides (Th, U) (Gu et al., 2017). However, the bioavailability of toxic metals and metalloids is usually low (Ruyters et al., 2011) and radioactivity below hazardous levels (Obhođaš et al., 2012). RM is discharged into the sea or specially constructed pools and pounds which may remain as caustic lakes long period after the plant closure (Obhođaš et al., 2012). The huge quantities of disposed material, RM high alkalinity of pH values 10-12.5 (Wang et al., 2008) and its high salinity, which may exceed 20 dSm^{-1} (Ruyters et al., 2011), are the main

concerns for the environment. The European Commission (EC) classifies RM as a mirror non-hazardous and a mirror hazardous waste (EC, 2018), while the US Environmental Protection Agency (EPA) RM classifies as non-hazardous waste (EPA, 1984). According to the EC Directive 2008/98/EC, a hazardous waste means a waste that displays one or more of the hazardous properties listed in Annex III (List of Waste, last amended by Commission Decision 2014/955/EU). The waste allocated to a mirror non-hazardous and mirror hazardous waste needs further steps in the assessment for allocation to either a hazardous or non-hazardous waste.

To date, the majority of ecological studies have been focused on toxicity effects of metals in RM (e.g. due to increased concentrations of V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Cd, Pb) (Brunori et al., 2005; Ruyters et al., 2011; Wang et al., 2008), but to our knowledge, no research has been conducted to understand the effect of the RM microscale particles on living organisms. Considering huge quantities of the RM disposed of the aluminum production plants and different RM recycling practices (e.g. usage of RM for soil remediation and wastewater treatment), potentially harmful microscale particles may be easily spread into the environment and eventually enter the food chain.

Earthworms are a good indicator of the overall soil status (Fründ et al., 2010) as they play an important role in processing soil nutrients, organic matter, and control of soil microorganisms. Also, they are very suitable for toxicological studies since potential harmful effects can be easily observed in a relatively short period of time. They may take metals directly from the soil by the absorption through the exterior vascularized epidermal surface (Lanno et al., 2004). Using the method of oral gluing, it has been shown that earthworms may live normally for days without consuming any food (Vijver et al., 2003). Accordingly, it was concluded that the dermal route is the uptake route of importance for nutrients and metals absorption (Vijver et al., 2003). Nevertheless, it has been shown that adsorption through the

epidermis is not a rate-limiting step in the metals uptake by earthworms (Vijver et al., 2005), but another intake channel together with the uptake via food ingestion. It is known that earthworms are quite resistant to the accumulation of toxic metals, possessing efficient storage and elimination mechanisms for their excessive concentrations (Ireland, 1977; Usmani and Kumar, 2015). However, the dermal absorption of metals may be lethal if the uptake is too fast for earthworms' regulatory mechanisms to adapt (Reinecke et al., 1997).

The goal of this research was to find the role of the RM microscale particles on the bioaccumulation of elements in earthworms. As a test organism, we have used the *Eisenia fetida*. This is an ultra epigeic species (Schultz and Joutti, 2007), which rests in soil and comes out to the surface to feed on decaying organic materials. The species has been reported to be tolerant of a wide range of pH and high conductivity and salinity values (Gunadi and Edwards, 2003; Owojori et al., 2008; Sharif et al., 2016). The presented results show that microscale particles in RM may impair the normal dermal functioning of earthworms, causing a decrease of almost all measured elements and potential deficiency in essential elements. The healthy earthworms (those not exposed to an overcritical amount of RM microscale particles) were able to mobilize and process the metals from the RM amended soils by their epithelium.

2. Materials and methods

2.1 Bioassay

Adult *Eisenia fetida* earthworms with well-developed clitellae were exposed to two types of RM: i) RM1, obtained from the abounded caustic basins near the closed aluminum processing plant in Obrovac, Croatia and ii) RM2, from the flooded area along the Torna creek, Ajka, Hungary, collected 4 months after a catastrophic event in 2010 when basin dams burst to release about 800.000 m^3 of slurry (Ujaczki et al., 2015). RMs were dried at room temperature, grounded and sieved to particle diameter < 2 mm.

Artificial soil was created according to guidelines for testing of chemicals no. 222: earthworm reproduction tests (OECD, 2004) using the 70% air-dried quartz sand, 20% kaolin clay, and 10% sphagnum peat. The pH value was adjusted to 6.0 ± 0.5 using the calcium carbonate. The soil moisture was set to 50% of water-holding capacity (OECD, 2004). Artificial soil was amended by adding RM1 or RM2 in increasing quantities: 4%, 8%, 12%, 16%, 20%, 40% and 60%. RM1-C and RM2-C correspond to the control samples made only of artificial soil. Soil mixtures were named RM1-4%, RM2-4%, etc., depending on the quantity of added RM. The chemical properties of RM1 and RM2 have been described elsewhere (Hackenberger et al., 2019; Obhođaš et al., 2012; Ruyters et al., 2011). Soils were mixed for 24 hours before the exposure tests. After mixing, pH and electrical conductivity were measured (Hackenberger et al., 2019). Before the beginning of the experiment, earthworms were placed on a moist filter paper to clear their guts. Seven earthworms were added to each glass container containing 500 g of soil amended with RM. Each exposure was done in triplicate. All containers were closed with a perforated lid and placed in the incubator under 16:8 light:dark ratio and constant temperature (20 ± 1 °C) for 7 days. Afterward, the containers were emptied, earthworms washed in distilled water, and placed on a moist filter paper to clear their guts. Earthworms were homogenized with a Potter-Elvehjem homogenizer on ice with a phosphate buffer saline (0.1 M, pH 7.4) in a ratio of 1:5 w/v and centrifuged at 15,000 G for 30 minutes at 4 °C. The bioassay experiment was done at the Department of Biology, University of Osijek, Croatia.

2.2 Analysis of soil samples

Soil mixtures to which earthworms were exposed were ground, homogenized and sieved to the particle diameter < 2 mm. Approximately 1 g of sieved samples were crushed in a mortar and sieved again (Φ = 45 μm) in order to press the pellets of 2.5 cm in diameter weighing about 1 g. Pellets were analyzed using the EDXRF. The analysis was carried out with

W anode and Mo secondary target in orthogonal geometry. Working parameters were set to 35 kV and 35 mA and irradiation time was 1000 s. X-ray spectra were collected with a Canberra Si(Li) detector with 3 mm thickness, 30 mm^2 active area and 0.025 mm Be window thickness, having a resolution of 170 eV (FWHM) at 5.9 keV. Spectra were analyzed using IAEA QXAS software. Concentrations of elements K, Ca, Ti, V, Cr, Mn, Fe, Ni, Cu, Zn, Ga, As, Br, Rb, Sr, Y, Zr, and Pb were obtained by direct comparison of count rates using the IAEA standard reference material Soil-7.

The passive radioactivity of the soil mixtures was measured on pellets prepared for the EDXRF analysis by analyzing U/^{226}Ra and Th. The coaxial Ge gamma detector with a relative efficiency of 20% was used. The detector was put in the shield composed of lead and iron in order to reduce the natural radioactivity coming from the background. Samples were analyzed individually for 22-25 hours. The activity and concentration of U/^{226}Ra and Th, respectively, were determined by using direct comparison with the IAEA standard reference materials (IAEA 313-Stream Sediment, IAEA 375-Soil, and IAEA 314-Stream Sediment). It has to be notified that U and ^{226}Ra activity cannot be distinguished by radiometric measurement.

2.3 Bioavailability analysis

Already prepared soil samples in the form of pellets were ground using the mortar and pestle. Approximately 1 g of each sample was weighed and put in the test tube and filled with 10 mL of double-distilled water (sample:water ratio of 1:10). Samples were left to incubate on the mixer for 24 hours (20 r/min). Subsequently, the samples were centrifuged for 20 minutes at 5000 r/min. The supernatant was separated into another test tube by a pipette and then vacuum filtered using the 0.45 μm cellulose nitrate filter paper. The pH value of the filtered supernatant was adjusted to approximately 3.5 by adding diluted nitric acid and/or ammonia. Ammonium pyrrolidine dithiocarbamate (APDC) 1% solution was freshly prepared and 1 mL

was added into each sample. Afterward, samples were left in the mixer for 20 minutes on 120 r/min. Mixed samples were then vacuum filtered through the 0.45 µm cellulose nitrate filter paper and analyzed using the EDXRF set-up as described above. The irradiation time was 500 s. Concentrations of elements Fe, Ni, Cu, Zn, and As were determined from the calibration lines prepared by Fluka liquid standard reference materials, 1000 mg/L. Calibration lines are shown in Supplementary materials.

2.4 Tissue analysis

Tissue samples were collected as the residue after centrifuging the earthworms. 1.5 mL of double distilled water was added to each sample after removing the supernatant for analysis of metallothionein content and enzyme activity. These results are presented elsewhere (Hackenberger et al., 2019). The amount of 10 µL of gallium solution was added as the internal standard. Samples were frozen using liquid nitrogen and lyophilized for 24 hours. Afterward, all the samples were transferred onto the 0.45 µm cellulose nitrate filter paper. Samples were analyzed using the EDXRF set-up as described above. The irradiation time was 500 s. Concentrations of elements K, Ca, Ti, V, Cr, Mn, Fe, Ni, Cu, Zn, As, Se, Br, Rb, Sr in tissues were obtained from the efficiency curve (see the Supplementary material) prepared by using Fluka liquid standard reference materials.

2.5 Feces analysis

Feces were collected from the moist filter papers on which the earthworms cleared their guts after the experiment. Before the analysis, the samples were preserved in the fridge. To each sample, 500 µL of double distilled water was added and they were left for two days in normal room temperature. Afterward, 50 µL of 1% APDC solution was added to aggregate the metals and the mixture was left for 20 minutes to stabilize. After stabilization, vacuum filtration was performed using the cellulose nitrate filter with a pore size of 0.45 µm. Samples were

analyzed using the EDXRF set-up as described above. The irradiation time was 500 s. Concentrations of elements K, Ca, Ti, V, Cr, Mn, Fe, Ni, Cu, Zn, Ga, As, Br, Rb, Sr, Y, Zr, and Pb were obtained by direct comparison of counts rates with the IAEA standard reference material Soil-7.

2.6 Particle size distribution analysis

Approximately 1 g of samples that were centrifuged for the bioavailability analysis were additionally dispersed in deionized water and briefly treated with ultrasound. For the laser diffraction, LS 13320, Beckman Coulter Ltd. was used with the working parameters of R.I. = 1.53, R.I.I. = 0.1, R.I.fluid = 1.33.

2.7 Earthworm pore size analysis using AFM

An earthworm was taken from the artificial soil, rinsed, frozen using liquid nitrogen, and left in a lyophilizer for 24 hours. It was glued to a metal disc and analyzed using Multimode AFM with Nanoscope IIIa controller (Bruker) and vertical engagement (JV) 125 µm scanner in contact mode. Sharpened silicon–nitride tips were used (NP-S, Bruker, nom. freq. 16-28 kHz, nom. spring constant of 0.12 N/m). The force was kept at the lowest possible value in order to minimize the forces of interaction between the tip and the surface. The linear scanning rate was optimized between 1.5 and 2 Hz with a scan resolution of 512 samples per line. Processing and analysis of images was carried out using NanoScope^{TM} software (Bruker, version V614r1). All images presented are raw data except for the first order two-dimensional flattening. Measurements were performed in air at room temperature and 50–60% relative humidity.

2.8 Earthworm pore size analysis using FE-SEM

The same earthworm used for AFM analysis was later investigated by the FE-SEM, JSM-7000F, under accelerating voltage of 1 kV, to confirm the AFM measurements of the

195 *E. fetida* microsize pores. After the results were confirmed, another earthworm exposed for 4
196 hours to soil mixture RM1-40% was frozen in liquid nitrogen without rinsing, lyophilized for
197 24 hours, and inspected using FE-SEM with the same working parameters. In addition, the
198 elemental composition of FE-SEM scanned microscale particles of the RM1-40% soil mixture
199 were analyzed by the Energy Dispersive X-Ray Spectrometry (EDS) under an accelerating
200 voltage of 10 kV, while images were scanned at 5 kV.

201 **2.9 Statistics**

202 The differences in distributions of results for bioavailability of RM1 and RM2 soil
203 mixtures were tested by using the Mann-Whitney U Test (ANOVA) at significant level
204 $p < 0.05$. Spearman Rank Order analysis at a significant level $p < 0.05$ and Kendall Tau analysis
205 at significant level $p < 0.1$ were used to check for correlations between concentrations of the
206 same elements in different matrices (soil concentrations, the bioavailability of soil elements,
207 tissue and feces concentrations). Statistics were calculated using Statistica 6 software.

208 # 3. Results

209 **3.1 Bioassay**

210 Earthworms survived all concentrations of added RM2. In the RM1 amended soils,
211 earthworms survived lower concentrations of RM, 14% earthworms died in RM1-40% and
212 only 14% survived in RM1-60% (Hackenberger et al., 2019).

213

214 **3.2 Soil and bioavailability analysis**

215 Soils containing the same quantities of RM1 and RM2 had similar pH value
216 (Hackenberger et al., 2019) in the range 7.07-8.13, which is suitable for the growth of the *E.*
217 *fetida* (Gunadi and Edwards, 2003). Measured radioactivity level with respect to U and Th

concentrations was highest in RM1-40% soil mixture (total was 1270 Bq), but well below the radiological risk for earthworms (Popic et al., 2012; Sheppard et al., 2005). The highest conductivity was found in RM1-60% soil mixture (1.296 d/Sm) (Hackenberger et al., 2019), which was well below the risk level for the earthworms' survival (Gunadi and Edwards, 2003; Sharif et al., 2016). The results of pH values, conductivity and radioactivity measurements are given in the Supplementary material. The content of 18 chemical elements was identified in soils amended with two kinds of RM. RM1 soil mixtures had higher concentrations of Ti, V, Cr, Mn, Fe, Ni, Sr, Y, Zr, and Pb, while RM2 soil mixtures had higher concentrations of K, Cu, Zn, Ga, Br, and Rb. The addition of both RMs resulted in metal concentrations increase, except for K and Rb, which concentrations were decreasing, and Cu and Zn, which concentrations were decreasing in RM1 mixtures but were approximately stable in RM2. Unlike in RM2 mixtures, Ga concentrations were decreasing and Sr concentrations were increasing with increasing quantities of added RM1. The figure showing concentrations diagrams of analyzed elements in soil mixtures is given in the Supplementary material.

Although concentrations of measured elements in RM1 and RM2 soil mixtures were statistically significantly different, the analysis did not show any significant statistical difference in the bioavailability of analyzed elements (Mann-Whitney U Test, $p < 0.05$). Fig.1 displays the diagrams of bioavailable concentrations of elements measured in RM1 and RM2 soil mixtures. Tables showing results of concentration levels in soil mixtures and their bioavailability are presented in the Supplementary material.

Figure 1

3.3 Tissues and feces analysis

The concentration of 15 elements were analyzed in the earthworm tissue (Fig.2). Earthworms exposed to RM2 had higher concentrations of nearly every measured element,

except Se and Br, which were higher in RM1 exposed earthworms, and Rb, which concentrations were similar in both RM1 and RM2 soil mixtures. A significant difference in earthworms' tissue was found by using Mann-Whitney U Test for concentrations of K, Ca, Ti, V, Cr, Mn, Fe, Ni, As, and Sr between earthworms exposed to RM1 and RM2 soil mixtures ($p < 0.05$). The results are shown in the Supplementary materials. Most of the concentration levels start decreasing at exposure to 40% and 60% of RM1 and at 60% of RM2. Concentrations of K and Rb in earthworm tissues were decreasing with the addition of increasing quantities for both RM1 and RM2 exposed earthworms.

Figure 2

The 18 elements were identified in the earthworm feces. Feces were only available from earthworms exposed to soil mixtures containing up to 20% of RM1 and up to 40% of RM2. Also, with the addition of larger quantities of RM, available feces were obtained in such a low amount enough to create only one sample for analysis. In general, concentrations of elements were higher in earthworms exposed to soil mixtures containing RM2. While concentration levels in feces were mostly increasing with increasing quantities of RM2, the trend was mostly negative for concentration levels in feces of earthworms exposed to increasing quantities of RM1. Tables with results of concentrations analyzed in earthworms' tissue and feces are presented in the Supplementary material as well as the figure showing diagrams of earthworms' feces concentrations.

3.4 Particle size distribution

Soil mixtures containing both types of RM consisted largely of microscale (0.1-1 μm) and nanoscale (< 100 nm) particles, however, RM1 amended soils had mostly twice as much microscale and nanoscale particles (in this study referred as microscale particles < 1 μm or

266 microscale particles) compared to samples with the addition of RM2. Consequently, RM1
267 amended soils had lower M_z (mean grain size) and D_{10}, D_{50}, and D_{90} values (intercepts for 10%,
268 50%, and 90% of the cumulative volume). Fig.3 presents particle size distributions analyzed in
269 soil mixtures with increasing quantities of RM1 and RM2. Tables presenting Mz, D_{10}, D_{50}, and
270 D_{90} values are given in the Supplementary material.

271 Figure 3

272 **3.5 Pore size analysis**

273 Size of the minute dermal pores of the *E. fetida* resting in the artificial soil was found to be in
274 100 - 460 nm range by AFM and in 90 - 420 nm range by FE-SEM, while dorsal pores were
275 fully closed. *E. fetida* exposed for 4 hours to soil amended with 40% of RM1, containing 31.6%
276 of microscale particles, was mostly attempting to escape from the container, vigorously trying
277 to get rid of the particles that were strongly adhered to its epithelium. The earthworm was fully
278 stretched for all the time of the exposure. The size of its dermal pores was determined to be in
279 315 - 735 nm range. Dorsal pores were fully opened, 75 x 43 μm in diameter, seeping huge
280 amounts of coelomic fluid. Minute dermal pores were found in the clusters irregularly
281 distributed along the earthworm epithelium. They were opened, partially closed, or completely
282 closed indicating active involvement in the normal physiological functioning of the epithelium.
283 Fig.4 shows the AFM image of the epidermal surface of the *E. fetida* exposed to control soil.
284 Fig.5 shows the FE-SEM image of *E. fetida* exposed to control soil and RM1-40% soil mixture.
285 FE-SEM images of RM1-40% soil mixture are presented in Fig.6 together with the results of
286 the Energy Dispersive X-Ray Spectroscopy (EDS) analysis.

287 Figure 4

288 Figure 5

289 Figure 6

290 **3.6 Correlation analysis**

291 The Spearman Rank Order correlation analysis for concentration levels of the same
292 element measured in different matrices (soil, the bioavailability of soil elements, tissue, and
293 feces) for RM2 amended soils confirmed significant correlations ($p < 0.05$) between soil/tissue
294 (Ca, Ti, V, Cr, Mn, Fe, As), soil/feces (Ca, Ti, V, Cr, Mn, Fe, Ni, Ga, As), tissue/feces (Ca, Ti,
295 V, Cr, Mn, Fe, Ni, As, Sr) and bioavailability/feces (-Zn). All correlations were positive except
296 the correlation found for Zn between bioavailability and feces. Very few correlations were
297 found for RM1 amended soils: tissue/feces (-Ti, -Cr, -Fe) and soil/feces (K, Cu, Rb).
298 Correlations found for tissue/feces matrices were all negative, while correlations between
299 soil/feces matrices were all positive. No correlations were found between soil and tissue for
300 RM1 soil mixtures. In addition, no correlations were found in soil/bioavailability and
301 bioavailability/tissue matrices for both RM1 and RM2 amended soils.

302 At a lower significant level ($p < 0.1$) and by using Kendall Tau analysis, correlations
303 were found between the amount of microscale particles < 1 μm and bioavailability for RM2
304 amended soils (-Zn, As). Bioavailability of Zn decreased while concentrations of As increased
305 with an increased amount of microparticles. More detailed results of the correlation analyses
306 are given in the Supplementary materials.

307

308 # 4. Discussion

309 **4.1 Uptake routes and bioaccumulation**

310 Soils amended with the same quantities of two kinds of RM used in this study have had
311 similar pH value and bioavailability of chemical elements, yet the earthworm mortality

312 occurred only in soils amended with RM1 containing higher quantities of microscale particles.
313 Surprisingly, the analysis of the earthworm tissue in our research revealed that the mortality in
314 earthworms exposed to RM1 did not occur because of the toxic effects. Instead, a decrease in
315 tissue concentration levels was noticed in earthworms exposed to RM1-40% (containing
316 31.6% of particles < 1 μm) and RM1-60% (containing 46.1% of particles < 1 μm) resulting in
317 14% and 86% mortality, respectively. Even though no earthworms died in RM2 soil mixtures,
318 a visible decrease in tissue concentration levels has occurred at exposure to RM2-60%
319 (containing 23.3% of particles < 1 μm). In addition, the observed lack of earthworms' feces
320 excretion in RM1-40%, RM1-60%, and RM2-60% and observed decreased excretion in RM1-
321 16% (containing 24.8 of particles < 1 μm), and RM1-20% (containing 33.2% of particles < 1
322 μm), indicate a slowing of the earthworms' metabolism at abundances of 23-25% of RM
323 particles < 1 μm.

324 RM has a large neutralizing and absorbance capacity, therefore the low bioavailability
325 of elements was presumed. However, no relationship has been found between bioavailability
326 and tissue, neither for earthworms exposed to RM1 or RM2 soil mixtures. Being an ultra
327 epigeic species it is highly unlikely that *E. fetida* feeds on soil particles. This has been
328 confirmed by Unrine et al. (2010) who did not find soil particles in the gut tissue regions of *E.*
329 *fetida* exposed to Cu ions and Cu nanoparticles. Considering this, it implies that earthworms
330 do not use exclusively the passive diffusion for dermal uptake of nutrients as suggested by
331 other studies (Sinha et al., 2008; Yu et al., 2006), but also active biodegradation of soil particles
332 for obtaining nutrients, metals in particular. Active biological reduction of Cu in soil has been
333 confirmed by Manceau et al. (2008) and discussed by Unrine et al. (2010). It is reasonable to
334 believe that other metals behave similarly.

335 Many researchers observed positive soil vs. tissue correlations in earthworms (e.g. for
336 Cr, Cu, Zn, Cd, and Pb) (Morgan and Morgan, 1988; Unrine et al., 2010; van Gestel et al.,
337 1993). Soil/tissue correlations for many elements have been observed in the present study, but
338 only in earthworms exposed to increased amounts of added RM2. The complete lack of
339 soil/tissue correlations observed in earthworms exposed to RM1 was surprising as well as
340 drastically reduced tissue and feces concentrations. The deficiency of essential elements
341 (Depledge and Rainbow, 1990) in earthworms is rarely reported (van Gestel et al., 2010). Most
342 reports quote regulated levels of Zn and Cu in *E. fetida* and *E. andrei* between 80 and 120
343 $mgkg^{-1}$ (van Gestel et al., 2010). However, these levels increase in highly contaminated soils.
344 Our observations show approximately constant concentrations of Cu (ranging between 20 and
345 50 $mgkg^{-1}$) and Zn (ranging between 100 and 140 $mgkg^{-1}$) in *E. fetida* tissue for both RM1 and
346 RM2 soil mixtures. The constant Cu and Zn concentrations in earthworm tissue were observed
347 regardless of decreasing Cu and Zn concentrations in RM1 amended soils. This confirms the
348 findings of other researchers that Cu and Zn accumulation in earthworms is physiologically
349 regulated (Morgan and Morgan, 1988; van Gestel et al., 1993). The As and Se (metalloids),
350 and especially Br (non-mental) were the only elements which concentrations were higher in
351 earthworms' tissue exposed to RM1-60% compared to control. Br as a halogen has a high
352 affinity for organic matter. Its increased tissue concentrations might be indicative for more
353 active oral intake of food by RM1-60% exposed earthworms whose dermal uptake was
354 impaired. The importance of both, oral and dermal intake of nutrients, is well established for
355 earthworms (Diez-Ortiz et al., 2015; Vijver et al., 2003, 2005). However, additional (as yet
356 undetermined) factors besides oral intake may be responsible for increased tissue
357 concentrations of As, Se, and Br which in RM1 exposed earthworms accumulated differently
358 than metals.

In contrast to essential trace elements Cu and Zn, which tissue concentrations remained stable throughout the trial, the essential elements K, Ca, and Fe are required in much higher concentrations. The earthworms exposed to RM1-60% were probably not able to compensate by oral intake the requirements for K, Ca, and Fe, resulting in drastically reduced tissue concentrations of these elements compared to control. The tissue concentrations of K, Ca and Fe in RM1-60% earthworms when compared to control decreased from 800 $mgkg^{-1}$, 1800 $mgkg^{-1}$ and 1900 $mgkg^{-1}$ to 400 $mgkg^{-1}$, 1200 $mgkg^{-1}$ and 270 $mgkg^{-1}$, respectively. It might be hypothesized that 86% mortality rate in this group has occurred because of the deficiency in these elements; although some other underlying mechanisms might occur, such as suffocation due to dermal desiccation, that cannot be discerned. It is worthwhile to notice the results of the study of *E. fetida's* avoidance of biochar amended soils (Li et al., 2011). The researchers have found that the increasing the water holding capacity of biochar amended soils from 85% to 100% might depress the negative effect of earthworm avoidance, most likely caused by desiccation (Li et al., 2011).

4.2 Link between deficiency and microscale particles

The lack of soil vs. tissue correlations in earthworms exposed to RM1 indicates impairment of dermal function, which has been reflected as decreased concentrations measured in earthworms' tissue after being exposed to RM1. This was not the case with RM2 exposed earthworms. RM2 soil mixtures never exceeded the total amount of 24% of micro-particles < 1 μm, whereas RM1 exposed earthworms exceeded this value starting from RM1-16%. Soil particles adhere more or less firmly to the earthworm epithelium depending on particle size. Earthworms adsorb chemical elements through the epithelium, either from the soil pore water or from the adhered particles. The adhered particles may be a nuisance to the earthworm's movement. In this research, we hypothesize that adhered microscale particles significantly interact with the earthworm epidermis either by interrupting their normal dermal respiration or

by desiccating epidermis, thus disabling uptake of essential elements by dermal absorption. Both processes would be followed by the slowing of the earthworms' metabolism and consequently reducing the concentrations of the essential elements with lethal outcome. Earthworm exposed to RM1-40% and scanned by FE-SEM had its dorsal pores fully opened, seeping huge quantities of the coelomic fluid. Its dermal pores were also fully opened, ranging from 315 to 735 nm in diameter, indicating earthworm suffering from suffocation and/or desiccation. From the observation of this strong response to physical irritation by microscale particles adhered to the earthworm epithelium, we concluded that decreasing in tissue concentrations and consequently mortality in RM1-40% and RM1-60% occurred because of the strong adhesion of microscale particles to the earthworms' epithelium and blockage of their minute dermal pores. These minute dermal pores presumably have a role in moistening and dermal respiration, thus enabling normal uptake of oxygen and metals by dermal absorption (Edwards and Lofty, 1977). It is possible that these minute dermal pores are directly involved in respiration, enabling more efficient uptake and transport of oxygen to epithelium vascular system by increasing the active surface and/or by increasing the partial pressure of oxygen at the epithelium surface/vascular interface.

Perhaps the observations made in the present study may explain the underlying mechanisms responsible for effects observed in studies of Laossi et al. (2010) and Van den Hoogen et al. (2019). In the Laossi et al. (2010) review article, it has been noted that earthworms enhance the plant growth, however; the effect was notable for sandy soils (generally nutrient-poor soils), but it was not observed in clayey soil (generally nutrient-rich soil). Furthermore, the recent study of Van den Hoogen et al. (2019) on the global abundance of nematode, another group of important soil organisms, has shown that the soil characteristics are the main drivers of the nematode global distribution, with further implications on global carbon cycling. The highest nematode abundances were found in the sub-Arctic region, while

abundances were relatively low in tropical regions (Van den Hoogen et al., 2019), where red clay soils prevail. Results presented in these studies indirectly show that soil texture indeed significantly influence the soil organisms such as earthworms and nematode.

5. Conclusion

Our results have shown that earthworms exposed to high amounts of microscale particles in RM amended soils were subjected to detrimental effects in response to physical irritation by microscale particles strongly adhered to their epidermal surface, thus causing blockage of earthworms microsize pores, desiccation of epidermis and prevention of normal dermal respiration and absorption of essential metals. Problems in the normal functioning of earthworms' metabolisms, observed as feces extraction decreasing or lacking, occurred at the exposure of earthworms to soils containing more than 20% of particles < 1 μm. The exposure of earthworms to soils containing 30% or more of particles < 1 μm increased the mortality risk of the *E. fetida* to 14%. The soil mixtures containing 46% of particles < 1 μm increased mortality risk to 86%. These effects have to be envisaged when planning RM discharging or recycling activities such is the RM soil amendment.

Conflicts of interest

There are no conflicts to declare.

Acknowledgments

We would like to express our gratitude to Eršek family for the donation of *E. fetida* culture (www.ersek.hr) that was used for AFM and FE-SEM analysis. A. V. acknowledges the support from the Croatian Science Foundation project DOK-01-2018.

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550 **Figure Caption:**

551 Figure 1. Bioavailable concentrations of elements Fe, Ni, Cu, Zn, and As analyzed in artificial
552 soils and soil mixtures containing increasing quantities of RM1 and RM2.

553 Figure 2. Concentrations of elements K, Ca, Ti, V, Cr, Mn, Fe, Ni, Cu, Zn, As, Se, Br, Rb, and
554 Sr measured in the tissue of earthworms exposed to artificial soils and soil mixtures containing
555 increasing quantities of RM1 and RM2. The horizontal line presents the level of concentrations
556 measured in earthworms exposed to RM1 artificial soil.

557 Figure 3. Particle size distributions analyzed in soil mixtures containing increasing quantities
558 of RM1 and RM2, (C-artificial soil/control).

559 Figure 4. AFM image of the epidermal surface and microsize pores of *E. fetida* exposed to
560 control soil: A. Position of the AFM probe taken by CCD camera; B. Surface plot of height
561 data (scan size 5µm x 5 µm, vertical scale 250 nm); C. Deflection image of the marked region
562 shown at a higher resolution (scan size 3µm x 3µm); D. Corresponding topographic profiles
563 along indicated lines.

564 Figure 5. FE-SEM image of *E. fetida*: A. A dorsal pore of the earthworm exposed to artificial
565 soil; B. A dorsal pore of the earthworm exposed to RM1-40% soil mixture; C. Epidermal
566 surface and microsize pores of the earthworm exposed to artificial soil; D. Epidermal surface
567 and microsize pore of the earthworm exposed to RM1-40% soil mixture.

568 Figure 6. FE-SEM images of different scales and results of EDS analysis of RM1-40% soil
569 mixture.

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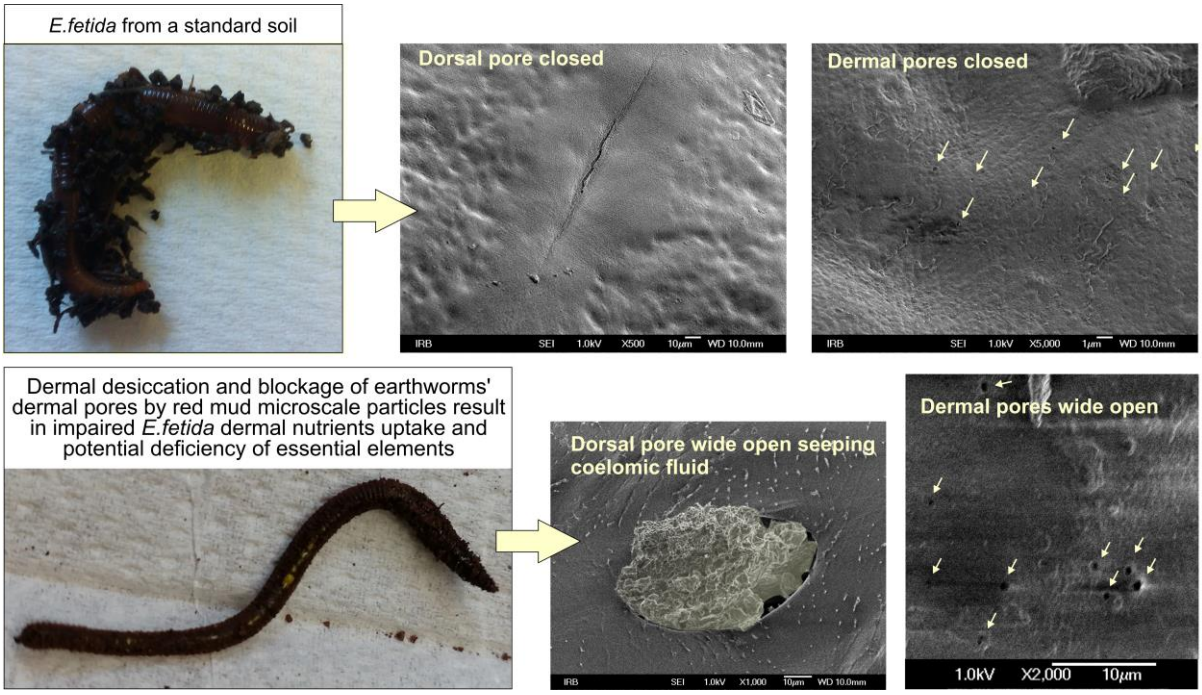

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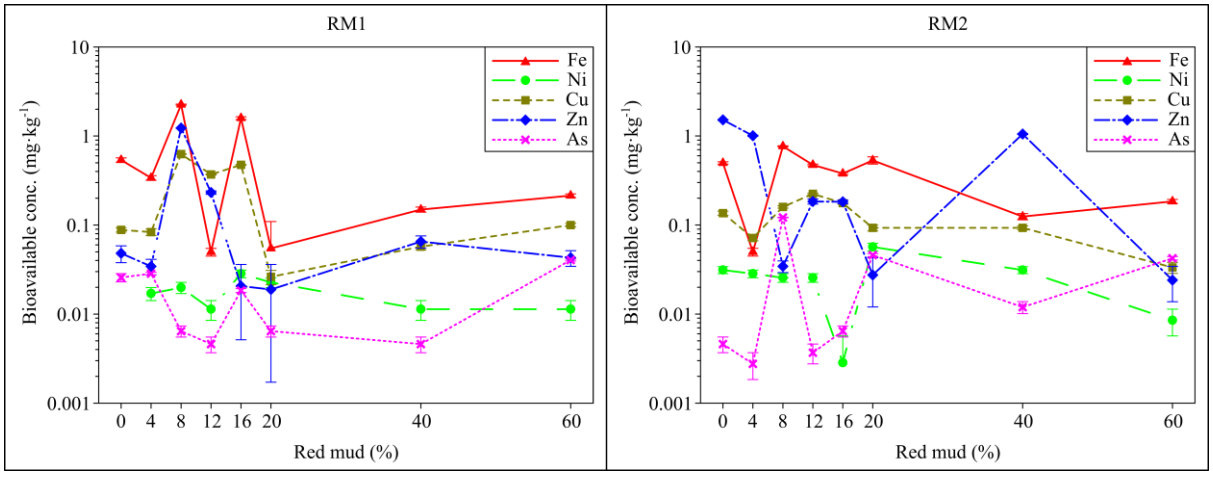
572 Graphical abstract



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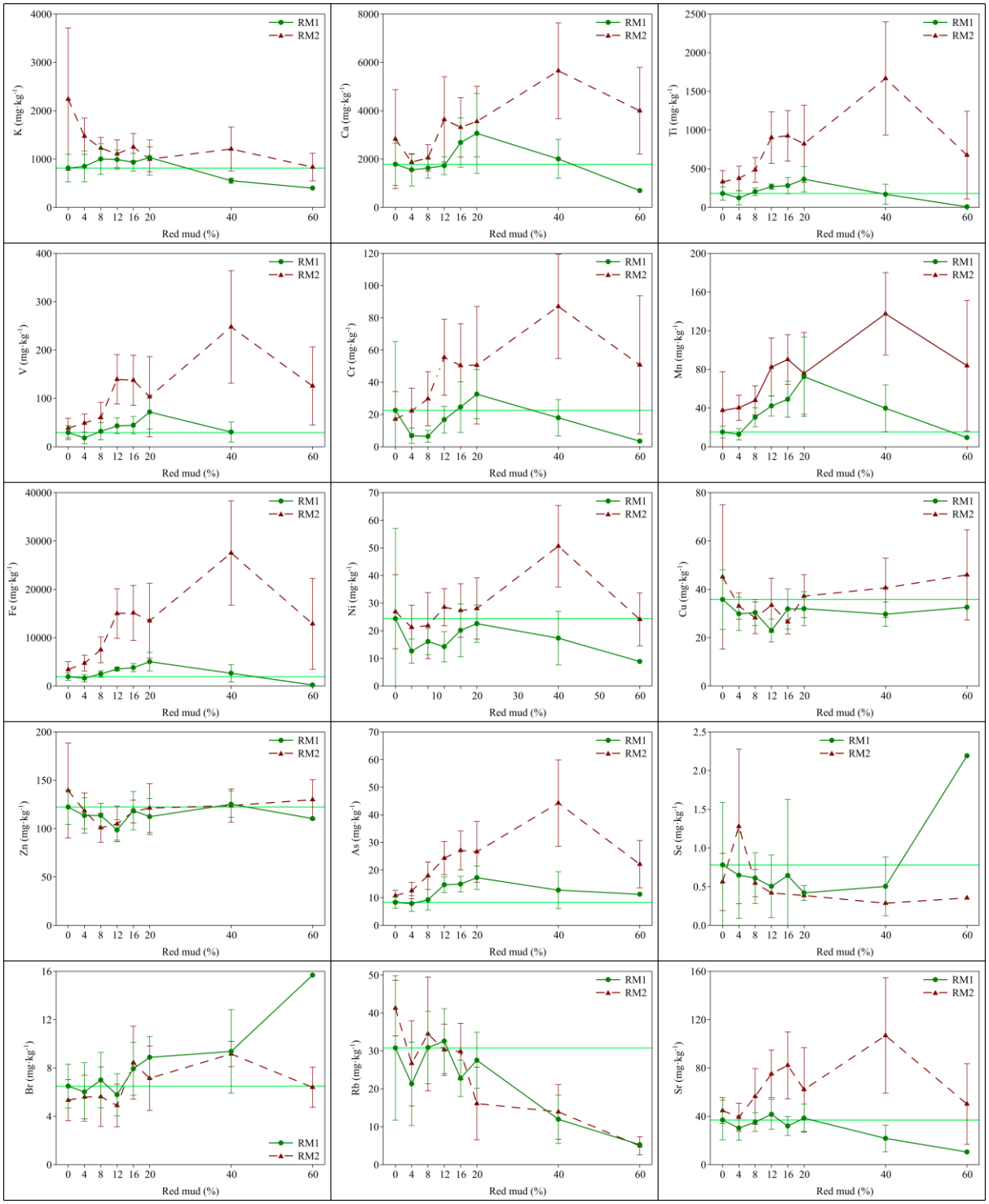

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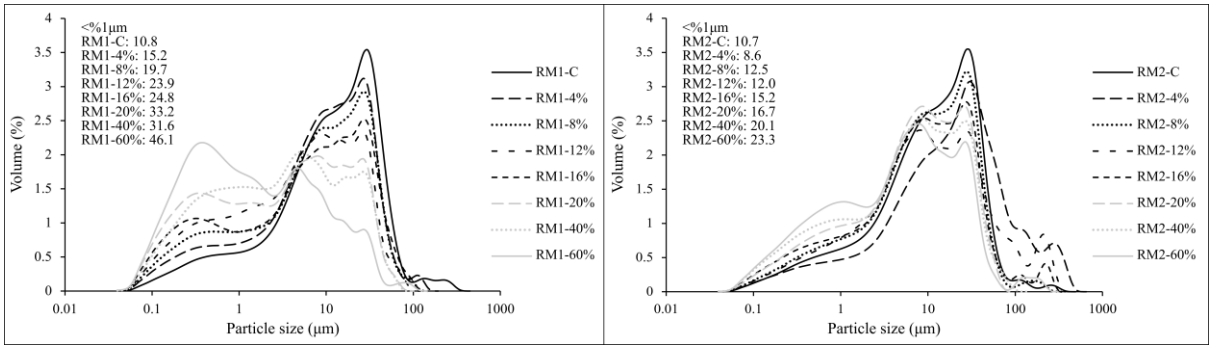

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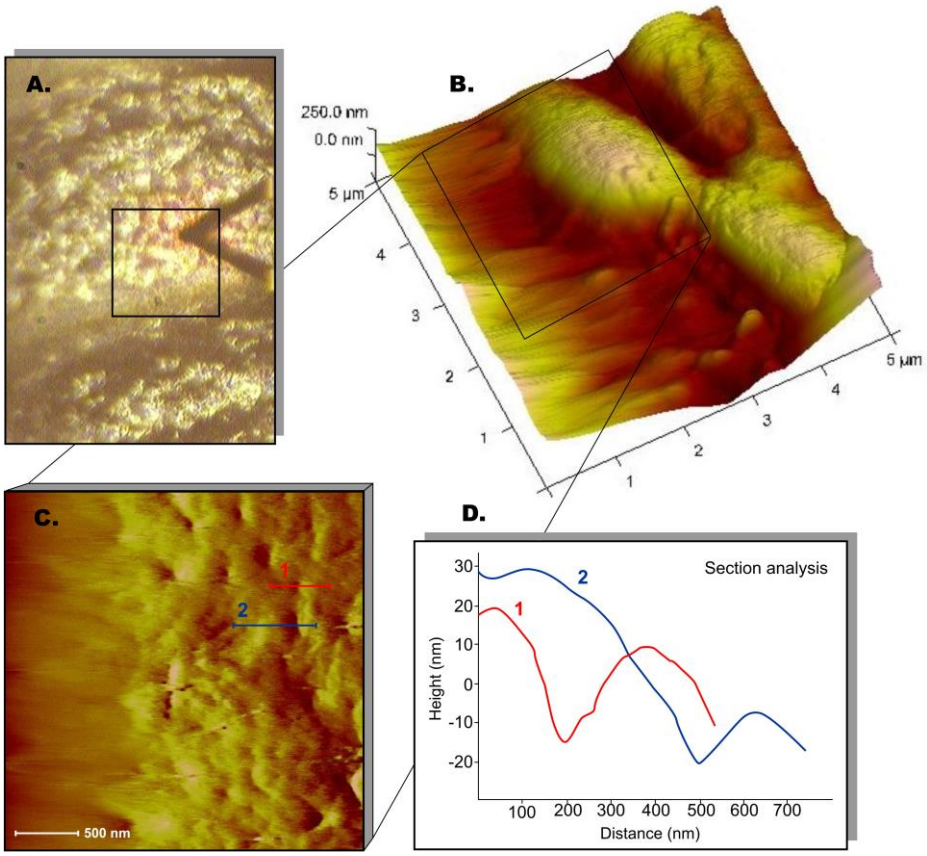

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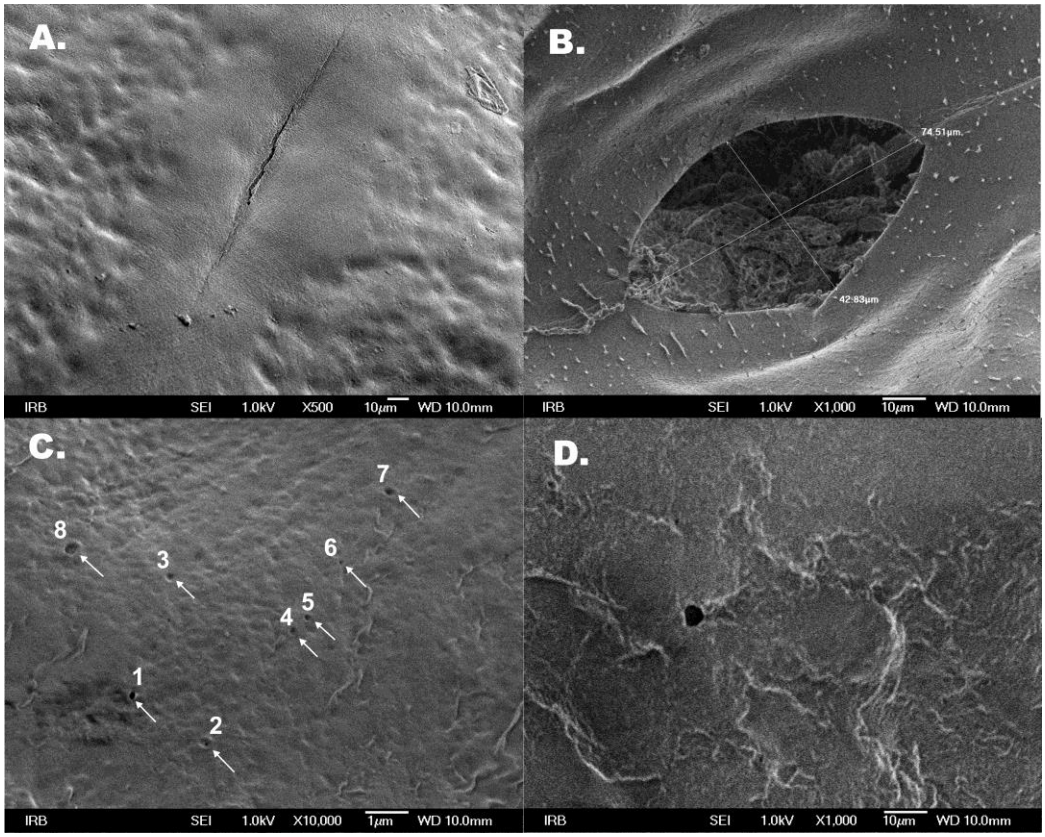

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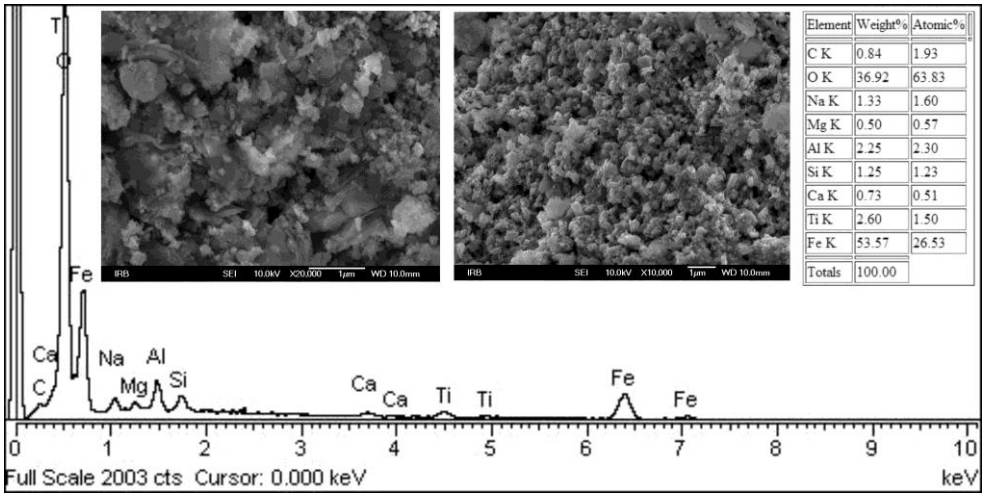

Element	Weight%	Atomic%
C K	0.84	1.93
O K	36.92	63.83
Na K	1.33	1.60
Mg K	0.50	0.57
Al K	2.25	2.30
Si K	1.25	1.23
Ca K	0.73	0.51
Ti K	2.60	1.50
Fe K	53.57	26.53
Totals	100.00	

Figure 6. FE-SEM images of different scales and results of EDS analysis of RM1-40% soil mixture.