

**Critical evaluation of the performance of rhamnolipids as surfactants for (phyto)extraction of Cd, Cu, Fe, Pb and Zn from copper smelter-affected soil**

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## ABSTRACT

Rhamnolipids are biosurfactants produced by bacteria belonging to the *Pseudomonas* genus. They are discussed to complex heavy metal cations stronger than cations of Fe, Ca, Mg. It is therefore suggested to employ rhamnolipids in phytoextraction where their addition to soil should result in preferential complexation of heavy metals that can be taken up by plants, thus enabling rapid and ecological clean-up of contaminated soil. In order to test this concept, we evaluated the rhamnolipid-mediated phytoextraction of heavy metal from soil collected from the vicinity of a copper smelter. The following aspects were investigated: i) selectivity of rhamnolipids towards Cu, Zn, Pb, Cd and Fe during soil washing; ii) phytoextraction efficiency of each ion with respect to the effective concentration of rhamnolipids; iii) possible phytotoxic effects; iv) effect of micro-sized polystyrene amendment. The experiments evaluated soil washing efficiency, BCR (Community Bureau of Reference) sequential extraction to determine the impact of rhamnolipids on the mobility of metal ions, phytoextraction with maize (*Zea mays* L.) and phytotoxic effects based on dry matter, chlorophyll fluorescence and content. The obtained results indicated that rhamnolipids lack desired selectivity towards heavy metal ions as Fe was complexed more efficiently by 80% of the available rhamnolipids compared to priority pollutants like Zn, Cu, Pb, which were complexed by only 20% of the tested rhamnolipids. With increased concentration of rhamnolipids, the soil washing efficiency increased and shifted in favour of Fe, reaching values of approx. 469 mg for Fe and only 118 mg in total of all tested heavy metals. Phytoextraction also favoured the accumulation of Fe, while Cd was not removed from the soil even at the highest applied rhamnolipid concentrations. Considering the selectivity of rhamnolipids and the costs associated with their production, our results suggest the need to search for other alternative (bio)surfactants with better selectivity and lower price.

## 1. INTRODUCTION

Rhamnolipids are natural glycolipid-based surfactants produced by several species of microorganisms, in particular by bacteria belonging to the *Pseudomonadaceae* family (Ławniczak et al., 2013; Szulc et al., 2014; Thakur et al., 2021). They are the most well-known and commonly studied anionic biosurfactants (Chrzanowski et al., 2012; Marecik et al., 2012). Their popularity results from their interesting properties (such as high surface activity, antibacterial action, modification of twitching and swarming phenomena as well as biodegradability) which correspond well with the concepts of green chemistry and a circular economy (Platel et al., 2020; Ren et al., 2023; Rikalovic et al., 2012; Romera-García et al., 2020). As a result, rhamnolipids have often been studied as a factor which can facilitate bioremediation attempts (e.g. enhanced biodegradation of petroleum contaminants in aquatic as well as terrestrial environments) (Chebbi et al., 2022; Dai et al., 2023; Mishra et al., 2023; Oyetibo et al., 2023; Posada-Baquero et al., 2019; Wilms et al., 2023; Zahed et al., 2022). In this regard, the frequently emphasized advantages of rhamnolipids are their exceptional effectiveness during soil washing or facilitation of phytoextraction in order to remove heavy metals such as Cd, Ni and Pb (Mulligan, 2005; Wang and Mulligan, 2004). According to many researchers, this is due to the fact that rhamnolipids form complexes with heavy metal cations more strongly compared to cations of common metals such as Fe, Ca, Mg (El Zeftawy and Mulligan, 2011; Lopes et al., 2021), making their action analogous to that of typical chelating agents used in phytoextraction (Gusiatin et al., 2021). This resulted in a concept that the addition of rhamnolipids to soil will result in preferential complexation of heavy metals, which will then be taken up by plants, thus enabling a rapid and ecological clean-up of contaminated soil (Jia et al., 2020; Liao et al., 2016; Liduino et al., 2018). However, despite much research regarding the complexation of heavy metals by rhamnolipids and their potential use for phytoextraction, there are still many issues that need to be explored further in order to clarify some inconsistencies and avoid potential bias during actual environmental clean-up attempts.

Firstly, the report regarding the complexation of rare earth elements using rhamnolipids highlights essential limitations in terms of selectivity (Hogan et al., 2017). The authors determined the complexation constants and divided the metals into three groups: ‘strongly bound group ( $\text{UO}_2^{2+}$ ,  $\text{Eu}^{3+}$ ,  $\text{Nd}^{3+}$ ,  $\text{Tb}^{3+}$ ,  $\text{Dy}^{3+}$ ,  $\text{La}^{3+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Al}^{3+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Y}^{3+}$ ,  $\text{Pr}^{3+}$  and  $\text{Lu}^{3+}$ ) with  $\log \beta$  ranging from 9.82 to 8.20. The elements  $\text{Cd}^{2+}$ ,  $\text{In}^{3+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Fe}^{3+}$ ,  $\text{Hg}^{2+}$ , and  $\text{Ca}^{2+}$  were moderately bound with  $\log \beta = 7.17\text{--}4.10$ . Finally,  $\text{Sr}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{UO}_2^{2+}$ ,  $\text{Ba}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Rb}^{+}$ , and  $\text{K}^{+}$  were weakly bound with  $\log \beta = 3.95\text{--}0.96$ ’ (Hogan et al., 2017). Based on these results, it can be assumed that  $\text{Fe}^{3+}$  will always compete very strongly with  $\text{Cd}^{2+}$ ,  $\text{Zn}^{2+}$  or  $\text{Hg}^{2+}$  ions. Consequently, whenever rhamnolipids will be used to remove such heavy metals, Fe ions may bind to the rhamnolipids and reduce their effective concentration.

Secondly, for rhamnolipids to form complexes with metals, they must be used in concentrations that compensate for the losses caused by the sorption of this biosurfactant in a given environmental niche.

In the very first report regarding the complexing properties of rhamnolipids, the authors clearly stated that: "Due to the strong sorption of rhamnolipid by soil, high levels of rhamnolipid treatment were required for efficient mobilization of soil-bound metals. At the lower concentrations of rhamnolipid tested (12.5 and 25 mM), sorption of rhamnolipid to soil was high (between 100 and 78% sorption) and seemed to limit the ability of rhamnolipid to desorb soil-bound metals (Herman et al., 1995)". Assuming that the average value of the critical micelle concentration ( $CMC_{water}$ ) for the system of the aqueous solution of rhamnolipids – air is equal to 100 mg/L, the 12.5 and 25 mM (used by the authors) translate to 7 and 14 grams of rhamnolipid. Notably, these values are 70 and 140 times higher than the  $CMC_{water}$ . The numerical value of  $CMC_{water}$  is applied to a standard aqueous solution – air system, which is significantly lower than the value of  $CMC_{soil}$ , which describes the critical micelle concentration for the aqueous surfactant – soil system (Parus et al., 2023). This occurs since the soil is a complex system of matter, in which several processes occur, such as the sorption of surfactants (in this case, rhamnolipids). Thus, this significantly influences the effective value of the CMC concentration."

This indicates two more challenges: the necessity to use higher concentrations of rhamnolipids in soil systems to ensure that complexation of metals may occur and the possible toxic effects that rhamnolipids may exhibit on the plant used for phytoextraction.

Moreover, it is a well-known fact that due to the widespread use of plastics, they are becoming one of the increasingly common components of soils. It is estimated that the typical plastic content of agricultural soils ranges from 0.05 to extreme value of 10% (de Souza Machado et al., 2019; Fuller and Gautam, 2016; Hüffer et al., 2019). According to many researchers, those polymers can affect the sorption and desorption processes, generally mobility, of organic but also inorganic contaminants (Cormier et al., 2022; Joo et al., 2021; Liu et al., 2022). Using polystyrene, defined in terms of particle size, pores and specific surface area, we set out to evaluate whether a model micro-polymer would exhibit a visible effect on rhamnolipids performance during removal of heavy metals from polluted soil.

All the above-mentioned problems were addressed in the framework of this study. The scientific aim was to evaluate the feasibility of rhamnolipid-mediated phytoextraction of heavy metal ions from soil located in the vicinity of a copper smelter, taking into account four major aspects: i) selectivity of rhamnolipids towards Cu, Zn, Pb, Cd and Fe during soil washing; ii) phytoextraction efficiency of each ion with respect to the effective concentration of rhamnolipids used for treatment; iii) possible phytotoxic effects; iv) effect of micro-sized polystyrene amendment (0.5% w/w).

## 2. MATERIALS AND METHODS

### 2.1. Chemicals

A commercially available rhamnolipids mixture, JBR-425 (25% aqueous solution), was obtained from Jeneil Biosurfactant Company (Saukville, Wisconsin, USA). The product mainly contains two rhamnolipid homologues: rhamnosyl- $\beta$ -hydroxydecanoyl- $\beta$ -hydroxydecanoate (RL1) and L-rhamnosyl-L-rhamnosyl- $\beta$ -hydroxydecanoyl- $\beta$ -hydroxydecanoyl- $\beta$ -hydroxydecanoate (RL2). Other chemicals used in this study for preparation of the heavy metal mixture, buffers, sample digestion, etc. were purchased from Sigma-Aldrich (Saint Louis, Missouri, USA) and Chempur (Piekary Śląskie, Poland).

### 2.2. Soil collection

Soil samples were collected from the vicinity of a copper smelter in Poland (Głogów, GPS coordinates: 51.6777, 15.9783), from a depth of 0-20 cm. Samples were then dried at 60 °C until constant weight was obtained. The samples were homogenized in the laboratory. The soil properties such as: metal content, pH, granulometric composition, porosity and sorption capacity were determined based on the methods described below. The soil is a fine-grained silt type OL with 2% clay, 67% silt and 31% sand. The following metals were detected: Zn 156 mg/kg, Cu 222 mg/kg, Pb 143 mg/kg, Cd 2 mg/kg, Fe 1836 mg/kg. Such heavy metal contents are typical for smelters which use polymetallic sulphides and were reported previously e.g. (Kabala and Singh, 2001). The macro and micro-element content in the soil was found to be: 21.3 mg K/kg, 314.7 mg Mg/kg, organic carbon content 1.2% (10.2 g/kg), N content 0.12%, 18.5 mg P/kg. The grain size distribution and particle size was determined to be: 1–0.1 mm = 31%, 0.1–0.02 mm = 67%, < 0.02 mm = 2%. The sorption capacity was 0.32 mL/g, porosity 0.4 mg/g and pH=6.35 in KCl, bulk density 1.46 Mg/m<sup>3</sup>, field water capacity 0.32 m<sup>3</sup>/m<sup>3</sup>, relative field capacity 0.80, moisture during sampling 16%, cation exchange capacity 23.6 cmolc/kg.

### 2.3. Granulometric composition of soil

The granulometric composition was analyzed using five sieves (Retsch, Haan, Germany) with the following sizes: 2 mm, 1 mm, 500  $\mu$ m, 250  $\mu$ m, 100  $\mu$ m, 45  $\mu$ m and 20  $\mu$ m according to (Mercier et al., 2001).

### 2.4. Soil porosity

Soil porosity was determined in a 100 mL cylinder. In the first stage, 30 g of dried soil, which corresponding approx. 30 ml in case of our soil, was introduced into the cylinder followed by 20 mL of distilled water. ~~In the first stage, 30 mL of soil were introduced into the cylinder followed by 20 mL of distilled water.~~

After each stage, the cylinder was weighed. In the next stage, a slurry was prepared using a glass stick and the cylinder was protected with parafilm. The sample was left for 24h to stabilize the phases and then the volumes of soil and water were measured (McCauley et al., 2009).

## **2.5. Sorption capacity**

Soil sorption capacity was determined in cylinders with 5 holes in the bottom. A paper filter was placed at the bottom of the cylinder and then weighed. Next, the soil was introduced into the measuring cylinder, in small portions, with gentle vibrations of 30 g of dried soil (which corresponds to a volume of 30 ml in the case of our soil). Then 20 ml of distilled water was added. The cylinder was secured from the top with parafilm and placed in a beaker into which the spill was collected. The sample was left for 2 weeks. The beaker was emptied of gravity water daily. When the gravity water stopped leaking from the cylinder, the cylinder was weighed to a constant weight (McCauley et al., 2009).

## **2.6. pH**

Determination of soil pH was carried out against 1 M KCl. To prepare the samples, 5 g of soil was added to a 25 ml conical flask, and 10 ml of 1 M KCl was added. The samples were shaken for 60 min and then filtered. The pH of the resulting aqueous filtrate was measured using a Metrohm titroprocessor 686 apparatus (Herisau, Switzerland) (McCauley et al., 2009).

## **2.7. Analysis of metals content in soil**

Metal concentrations in the soil (Cu, Cd, Zn, Pb, Fe, P, Ca, Na, Mg, K) were determined by digestion with acidic (73% HF, 65% HNO<sub>3</sub> and 30% H<sub>2</sub>O<sub>2</sub>) solutions, according to the technical protocol developed by (Lukaszewski et al., 2012). The digests were filtered through 0.22 µm PTFE filter (Whatman® Puradisc, Sigma Aldrich) into 25 mL flasks. The digested samples were analyzed for the metals by ICP OES (for more information please see section 2.14.).

## **2.8. Determination of critical micelle concentration in water and soil**

Air-water surface tension ( $\gamma$ ) was measured at 25 °C using an Attension Theta optical Tensiometer (KRÜSS, Hamburg, Germany). Surface tension was measured using freshly prepared aqueous solutions of rhamnolipids in water and soil solutions over a concentration range from 0.005 to 10,000 mg/L at 20 °C. The rhamnolipid content in the soil was determined after shaking 10 g of soil with 2 mL of each respective rhamnolipid solution. Surface tension was determined by the hanging drop method, following the procedure described previously (Parus and Framski, 2018).

## **2.9. Preparation of microplastic its characteristics and sorption experiment**

A description of the preparation and characterization of polystyrene (PS) microplastic is detailed in (Lisiecka et al., 2023). In brief, polystyrene (PS) with the trade name Empera 124L (Brenntag Polska Sp. z o.o., Poland) was milled using an ultra-centrifugal mill (ZM 200, Retsch, Haan, Germany) with a

vibrating feeder (DR 100, Retsch, Haan, Germany) in dry ice to prevent the plasticization of the polymer, as well as to increase the milling efficiency. The PS characteristics obtained were: average size of 276  $\mu\text{m}$ , low BET (Brunauer-Emmett-Teller theory) surface area (0.2  $\text{m}^2/\text{g}$ ), low total pore volume (0.001  $\text{mL/g}$ ) and average pore diameter of 23.1 nm for each granule. A short experiment was carried out to test whether heavy metals sorb onto the surface of PS. The experiment was carried out according to the stipulated guidelines (OECD/OCDE 106, 2000). A mixture containing: Zn(II) 21.5 mg, Cu(II) 39.6 mg, Pb(II) 2.6 mg and Fe(III) 335.4 mg was prepared in 50 mL glass vial. The amounts of heavy metals were selected according to the concentrations of metals in the leachable layers (soluble and exchangeable) after the BCR (European Community Bureau of Reference) sequential extraction tests (please see section 2.10.). After that, 25 mL of the heavy metal solution was mixed with 0.5% PS w/v. The glass vial was shaken on an orbital shaker at 240 rpm at  $20 \pm 1$  °C in the dark (in order to avoid any possible photodegradation). The sample was collected after 24 h and filtered through a 0.22  $\mu\text{m}$  PTFE syringe filter (Whatman® Puradisc, Sigma Aldrich, UK). The sample was analyzed for the metals by ICP AES (for more information please see section 2.14.). Reference trials were also carried out to check whether the sorption of heavy metals in polyethylene containers occurred. The results are presented in Table S1 (Please see ESI for more information). Notably, no statistically significant sorption was obtained for the metals tested.

## **2.10. Analysis of metal mobility in soil – BCR sequential extraction**

Laboratory experiments were conducted to determine the influence of rhamnolipids and PS on the mobility of major metals (Zn, Cu, Pb, Cd and Fe) present in the contaminated soil. Rhamnolipids were added at concentrations  $\text{CMC}_{\text{soil}} = 500$  mg/L,  $2 \times \text{CMC}_{\text{soil}} = 1000$  mg/L,  $3 \times \text{CMC}_{\text{soil}} = 1500$  mg/L based on previously determined critical micellar concentration for this soil type (Parus et al., 2023). Furthermore, tests were performed with the addition of 0.5% PS (w/w) to the soil and soil with the rhamnolipids at a concentration of  $2 \times \text{CMC}_{\text{soil}}$ .

Samples were shaken at 25 °C for 16 h, and then dried to constant weight at 60 °C. Afterwards, the soil samples were analyzed using the BCR sequential extraction method with the modifications done by (Jakubowska et al., 2008) and (Wali et al., 2015). The samples without rhamnolipids were used as a reference.

In the first step, the metals which dissolved in water, were extracted. Samples of each soil (1 g) were placed in the plastic containers and 40 mL of water were added. The samples were mechanically shaken (SK-O330-PRO, Chemland, Poland) at 240 rpm for 16 h. The extracts were separated from the solid residue by centrifugation (Centrifuge 5804, Eppendorf, Germany) at 10 000 rpm for 10 min and then collected in polyethylene containers.

In the second step, the exchangeable and soluble fractions were extracted. Then, 40 mL of 0.11 mol/L acetic acid solution were added to the residual soil samples. In the following step, the samples were

mechanically shaken end-over-end at 280 rpm for 16 h. The extracts were separated from the solid residue by centrifugation at 10 000 rpm for 10 min and collected in polyethylene containers.

In the third step, 40 mL of 0.5 mol/L hydroxyl ammonium chloride solution (pH 1.5 with HNO<sub>3</sub>) were added to the reducible fraction, and the mixture was re-suspended by mechanical shaking for 16 h.

In the fourth step, the oxidizable fraction was extracted. The samples were treated with 10 mL of 30% H<sub>2</sub>O<sub>2</sub> solution and the mixture was digested for 1 h at 85 ± 2 °C in teflon tubes on a ceramic plate (Chemland, Poland). The mixtures were evaporated to a volume lower than 3 mL. A second aliquot of 10 mL of 30% H<sub>2</sub>O<sub>2</sub> was added to the samples, which were shaken for 1 h at 85 ± 2 °C. Afterwards, the mixture was evaporated to 2 mL. The residues were treated with 50 mL of 1 mol/L ammonium acetate solution, adjusted to pH 2 with HNO<sub>3</sub> and shaken for 16 h. The extracts were separated.

In the fifth step, the solid residues were digested using an acidic (73% HF, 65% HNO<sub>3</sub>, 30% H<sub>2</sub>O<sub>2</sub>) solution. Three replicates were performed in parallel for each sample and blanks were measured for each set of analyses using acid extraction and BCR procedures, respectively. An internal check was performed on the sequential extraction results by comparing the total amounts of metals extracted by different reagents during the sequential extraction procedure with the results obtained by total digestion. The samples were analysed by ICP OES (Please see ESI for more information).

## **2.11. Soil washing with rhamnolipids**

The washing of Zn, Cu, Pb, Cd and Fe in the presence of rhamnolipids was evaluated in batch experiments. Namely, 2 g of dried and homogenized soil sample was added to 50 mL polyethylene centrifuge tubes, followed by 40 mL of rhamnolipid solution (CMC<sub>soil</sub>= 500 mg/L, 2×CMC<sub>soil</sub>=1000 mg/L, 3×CMC<sub>soil</sub>=1500 mg/L). Deionized water was used as a reference. Tests were also performed for soil mixed with PS (Soil + 0.5 % PS w/w). The tubes were shaken at room temperature for 16 h on a rotary shaker at 240 rpm (SK-O330-PRO, Chemland, Poland). The samples were then centrifuged at 10,000 rpm for 10 min (Centrifuge 5804, Eppendorf, Germany) and transferred to polyethylene containers. The metal content in the supernatant was determined by ICP OES (inductively coupled plasma – optical emission spectroscopy) (Please see ESI for more information).

## **2.12. Phytoextraction of heavy metals with rhamnolipids**

Phytoextraction studies were conducted using a greenhouse experiment with maize (*Zea mays* L.) as a model plant in 2 kg plastic pots filled with soil from a vicinity of a copper smelter in Poland (Głogów). The maize was grown for 3 weeks. Rhamnolipids were introduced at concentrations of CMC<sub>soil</sub>= 500 mg/L, 1.5×CMC<sub>soil</sub>= 750 mg/L, 2×CMC<sub>soil</sub>= 1000 mg/L. The rhamnolipid solutions were dosed one week after sowing the maize, when after the seeds had already germinated.



Control samples were watered with demineralized water. A 12/12 photoperiod was established in which the plant was exposed to light for 12 hours and maintained at 20 °C. Then, for the next 12 hours the plant had no light and the temperature was set at 17 °C. The soil moisture was set at 70% of field capacity and kept constant during the experiment. All experiments were performed in two repeats. After the experiment, the plants were separated from the soil, divided into roots and shoots, and dried to a constant weight at 40 °C. The soil was collected into polyethylene bags. The metal content (Zn, Cu, Pb, Cd and Fe) in the soil, roots and shoots was determined after digestion using an acidic solution (73% HF, 65% HNO<sub>3</sub>, 30% H<sub>2</sub>O<sub>2</sub>) by ICP OES (for more information please see section 2.14.).

The bioconcentration factor (BCF), the translocation factor (TF) and the enrichment factor (EF) were determined according to procedure provided by Nirola et al. (Nirola et al., 2015). BCF is the ratio of the metal content in roots to its content in soil (mg/kg). The translocation factor (TF) was calculated by dividing the metal content in shoots by its content in roots (mg/kg), while the enrichment factor (EF) was calculated as the ratio of the metal content in the shoots to its content in the soil (mg/kg).

### **2.13. Phytotoxic effects towards maize after phytoextraction**

After 21 days of phytoextraction tests, chlorophyll fluorescence was measured using Multi-Mode Chlorophyll Fluorometer (OS5p, Opti-Sciences, Inc., Hudson, NY, USA) with PAR Clip according to the instruction manual in Plant Stress Measurements. Fluorescence measurements were done in triplicate for each replication for one series of tests. Before the measurements, the leaves were dark-adapted for 30 minutes using white clips to silence photosynthesis. The following parameters were assessed: electron transport efficiency (ETR), initial fluorescence (F<sub>0</sub>), maximum fluorescence (F<sub>m</sub>), fluctuating fluorescence (F<sub>v</sub>), and photosystem efficiency (Y). For initial fluorescence, the light intensity was set to approximately 0.1 µmol. The chlorophyll content (CCI) was determined by measuring the optical absorbance at 620 nm.

## **3. RESULTS AND DISCUSSION**

### **3.1. Soil washing with rhamnolipids**

The first experimental step was focused on the analysis of selectivity during the removal of heavy metal ions by washing soil from the vicinity of a copper smelter with rhamnolipid solutions at various concentrations. Based on the data presented by Hogan et al. (Hogan et al., 2017), it was expected that rhamnolipids will not exhibit selectivity towards heavy metals. The obtained results were presented in Table 1. It can be observed that the addition of rhamnolipids, depending on the concentration, increased Cu leaching by 2.6, 3.9 and 5.2 times, Zn leaching by 4.8, 6.5 and 8.1 times, and Pb leaching by 4.9, 7.7 and 9.0 times compared to deionized water. Interestingly, no leaching of Cd was observed. A general

analysis of all the relevant metals in the analyzed soil indicated that the Fe was the most abundant, one order of magnitude higher than the sum of all other heavy metals. Accordingly, Fe was also complexed by rhamnolipids and leached 6.4, 9.3 and 12.8 times better in comparison to deionized water, respectively.

**Table 1.** Effect of rhamnolipid introduction on metal washing from soil sampled from the vicinity of a copper smelter.

Washing Agent	Cu [mg/kg]	Zn [mg/kg]	Pb [mg/kg]	Cd [mg/kg]	Sum of heavy metals [mg/kg]	Fe [mg/kg]
<b>Soil</b>						
<b>Initial metal concentration</b>	222.1 ± 2.3	156.3 ± 1.8	143.4 ± 0.7	2.1 ± 0.2	523.5 ± 5.0	1836.3 ± 1.7
<b>Deionized water</b>	16.3 ± 0.2	0.0 ± 0.0	2.8 ± 2.2	0.0 ± 0.0	19.1 ± 2.4	36.7 ± 1.4
<b>Rham at CMC<sub>soil</sub></b>	41.5 ± 0.6	4.8 ± 0.5	13.5 ± 1.9	0.0 ± 0.0	59.8 ± 3.1	233.7 ± 1.1
<b>Rham at 2×CMC<sub>soil</sub></b>	63.1 ± 0.1	6.5 ± 1.7	21.3 ± 1.1	0.0 ± 0.0	90.9 ± 2.9	341.2 ± 1.6
<b>Rham at 3×CMC<sub>soil</sub></b>	84.2 ± 0.7	8.1 ± 1.3	25.2 ± 1.3	0.0 ± 0.0	117.5 ± 4.2	468.7 ± 3.8
<b>Soil + 0.5 % PS w/w</b>						
<b>Deionized water</b>	15.4 ± 0.4	0.0 ± 0.0	1.9 ± 1.2	0.0 ± 0.0	17.3 ± 1.7	37.3 ± 1.8
<b>Rham at 2×CMC<sub>soil</sub></b>	61.9 ± 0.3	5.7 ± 1.2	22.1 ± 0.7	0.0 ± 0.0	89.3 ± 3.1	337.1 ± 2.2

± Relative value of the standard deviation (RSD)

\*Rham – rhamnolipids

The obtained results confirm the hypothesis regarding a lack of selectivity towards heavy metal ions, as the preferential complexation of Fe was observed during rhamnolipid-assisted metal leaching from a soil contaminated within years of copper smelter operation in the area. The sum of initial concentrations of Zn, Cu, Cd and Pb was equal to 523 mg, which is more than three times lower in comparison to the Fe content (1836 mg). The rhamnolipids leached a total of 60 mg of heavy metals from the soil compared to 234 mg of Fe at the CMC<sub>soil</sub> biosurfactant concentration, and 91 mg of heavy metals and 341 mg of Fe, respectively, for the 2×CMC<sub>soil</sub> concentration. Assuming that a minimum of one rhamnolipid molecule is needed to complex the analysed ions, 3.9 and 3.8 times more rhamnolipids were used to complex Fe than the more environmentally relevant heavy metals for the 2×CMC<sub>soil</sub> and 3×CMC<sub>soil</sub> biosurfactant concentrations, respectively. Therefore, it can be concluded that only 20% of the applied rhamnolipids worked as expected, while 80% were bound by a metal which is relatively abundant in soils and non-essential for environmental clean-up purposes. In strictly economic terms for every \$100 invested in such a process, only \$20 is profitable and \$80 is a loss, which results from the lack of selectivity that commercial chelating compounds have. In consequence, it seems that, despite their many merits, rhamnolipids may not be an optimal solution for *in situ* removal of heavy metals from soil, since synthetic compounds are much cheaper and also more selective (Chong and Li, 2017; Jiang et al., 2021; Lotfabad et al., 2016) (Table 2).

Table 2. Cost of rhamnolipids biosurfactant compared to typical synthetic chelators reported for phytoextraction

Commercial surfactant	Type of agent	Price \$ per kg*	Producent	Reference
R90, 85-90% pure rhamnolipids	biosurfactant	1250	AGAE Technologies LLC, (Corvallis, OR, USA)	(“ <a href="https://www.agaetech.com/collections/r-90-grade/products/r90-1kg">https://www.agaetech.com/collections/r-90-grade/products/r90-1kg</a> ,” 2023)
NTA, ACS reagent, ≥99.0%	synthetic chelator	182	Sigma-Aldrich (Saint Louis, MO, USA)	(“ <a href="https://www.sigmaaldrich.com/US/en/product/sial/72560">https://www.sigmaaldrich.com/US/en/product/sial/72560</a> ,” 2023)
EDTA, ACS reagent, 99.4-100.6%	synthetic chelator	252	Sigma-Aldrich (Saint Louis, MO, USA)	(“ <a href="https://www.sigmaaldrich.com/US/en/product/sial/e9884">https://www.sigmaaldrich.com/US/en/product/sial/e9884</a> ,” 2023)
DTPA, for complexometry, ≥99.0%	synthetic chelator	664	Sigma-Aldrich (Saint Louis, MO, USA)	(“ <a href="https://www.sigmaaldrich.com/US/en/product/sigma/32319">https://www.sigmaaldrich.com/US/en/product/sigma/32319</a> ,” 2023)
IMDA, 98%	synthetic chelator	122	Sigma-Aldrich (Saint Louis, MO, USA)	(“ <a href="https://www.sigmaaldrich.com/US/en/product/aldrich/220000">https://www.sigmaaldrich.com/US/en/product/aldrich/220000</a> ,” 2023)

\* Current prices from 26<sup>th</sup> July 2023, NTA- Nitrilotriacetic acid, EDTA- Ethylenediaminetetraacetic acid, DTPA- Diethylenetriaminepentaacetic acid, IMDA- Iminodiacetic acid

Similar conclusions were established by Lu et al. as the authors indicated that heavy metal leaching using rhamnolipids, in combination with phytoextraction, is a less efficient approach compared to commercial chelating agents, such as EDTA or EDDS (Lu et al., 2017).

The results for soil and soil + 0.5% PS (w/w) addition were very similar and did not differ notably, indicating that the addition of this type of microplastic had no effect on metal washing and, at the same time, did not play a key role in metal sorption processes in the analysed soil. The polystyrene microplastic added into the system with rhamnolipids, at a concentration of  $2 \times \text{CMC}_{\text{soil}}$ , also did not play a significant role (data not shown).

The sorption of heavy metals themselves onto microplastics is the subject of numerous studies by scientists (Wang et al., 2020; Yang et al., 2019a; Zou et al., 2020). They point out the important role played by the surface area of a given microplastic, as well as the sorption mechanisms, which include, for example, electrostatic interaction and surface complexation, physical and chemical adsorption or Van der Waals forces (Guo et al., 2020; Lang et al., 2020a; Shen et al., 2021). Notably, sorption is dependent on whether one is dealing with a pristine or aged polymer, due to the fact that aged polymers have an increased: availability of sorption centers, number of sorption groups and surface area. In

addition, aged polymers more realistically reflect the environmental conditions (Lang et al., 2020a; Mao et al., 2020; Wang et al., 2019).

In the case of pristine polymers, sorption generally does not occur, as in our study, or is low, as shown, for example, by Lang et al. where a Cd sorption of 0.02 mg/g on pristine PS was reported (Lang et al., 2020b). In other studies, it was found that pristine PS with surface area  $<0.001 \text{ m}^2/\text{g}$  able to sorb 0.008 mg/g Cu (Yang et al., 2019b). Other studies indicated that PS with an area of  $3.2 \text{ m}^2/\text{g}$  was able to sorb 0.81 mg/g Co, 0.47 mg/g Cr and 0.36 mg/g Cu (Godoy et al., 2019). Studies with aged polymers show, for example, that aged, beached pellets accumulate heavy metals to a much greater extent than virgin pellets (Holmes et al., 2012). Other studies have compared the sorption of heavy metals on unaged and aged PS using the Fenton and  $\text{H}_2\text{O}_2$  aging. The result was that the sorption of heavy metals onto PS was significantly enhanced after aging (Lang et al., 2020a).

It should also be noted that sorption efficiency is mainly influenced by soil type. Thus, the addition of a pristine microplastic with a poor porous structure does not have a statistically large effect on soil leaching, washing out and the sorption of heavy metals in the soil thereof.

### 3.2. Analysis of metal mobility in soil – BCR sequential extraction

In order to further investigate this issue, the effect of rhamnolipids on the mobility of metals in soil was analysed using BCR sequential extraction (Parus and Framski, 2018; Paul, C.J., Ford, 2000), which allowed to precisely determine whether the studied metals translocate between different soil fractions and to what extent they do so (Figure 1).

**Figure 1.** BCR fractions of Zn, Cu, Cd, Pb, Fe. Description of the fraction: **sol**- soluble, **exch**-exchangeable, **red**- reducible, **oxi**- oxidizable, **res**- residual. (different letters indicate statistical difference)

In the analysed soil, the highest amounts of metals were associated with soil organic matter and sulphides. The introduction of rhamnolipids into the soil had little effect on the translocation of Zn, Cu and Cd between the analysed fractions, and the highest metal concentrations were observed in the residual fraction. This observation suggests the incorporation of Zn, Cu, Cd into insoluble minerals. Many authors refer to metals in the residual fraction as only available through digestion with strong acids (Jiang et al., 2021; Wali et al., 2015). Although the solubility constants will change when complexing agents are present in the system, a significant effect of rhamnolipids in this regard should not be expected (Jensen et al., 2011). The results obtained in this part of the study correspond well with

the reports of other researchers. For example, (Jensen et al., 2011) indicated that due to their very limited ability to solubilize Cu and Zn, rhamnolipids cannot be recommended for use in enhanced phytoextraction of heavy metal-contaminated soils, at least not for calcareous soils.

Curiously, Pb reacted to the addition of rhamnolipids in an interesting manner, as an almost quantitative transfer from the residual to the reducible fraction occurred. Considering the fact that Pb compounds present in mine tailings exhibit much lower mobility compared to metals such as Zn, Cu or Cd (T. G. Lovering, 1976), the obtained result is intriguing. However, a similar behaviour of Pb in the presence of rhamnolipids has already been described in the study of (Maier et al., 2001). The mechanism of such action of rhamnolipids certainly needs to be examined in detail, but it is beyond the scope of this study.

Of highest interest, however, is the behaviour of Fe after the introduction of rhamnolipids. The Fe content increased in the exchangeable, reducible, and oxidizable fractions. In general, metals present in the soluble and exchangeable fractions are mobile, whereas metals in the oxidizable and reducible fractions could potentially become mobile when environmental conditions change (Borgese et al., 2013; Hu and Cheng, 2016). Since rhamnolipids are susceptible to sorption in the soil, a possible scenario is that the adsorbed rhamnolipids will not be biodegraded, and if soil parameters change, they will be released with the next portion of heavy metals, perhaps in an uncontrolled manner, yet this aspect requires further research. It is also worth mentioning that numerous reports which indicated a positive effect of rhamnolipids on heavy metal removal were based on experiments in which heavy metals were introduced into the soil in the form of soluble salts (Lu et al., 2017; Mekwichai et al., 2020; Wen et al., 2010). Under real conditions, heavy metals enter the soil and bind to the soil matrix in a very complex way. Many research reports and many excellent reviews have been devoted to these issues (Guala et al., 2010; Markiewicz-Patkowska et al., 2005; Uddin, 2017). Prolonged residence of a metal in the soil usually causes a significant reduction of its bioavailability precisely due to binding with the soil matrix. This effect is omitted when soluble salts are used, since all such interactions that hinder metal leaching from the soil are eliminated which may lead to the overestimation of the effect of rhamnolipids. It is well known that the soluble fraction contains the elements that are most readily available to plants and that are able to penetrate rapidly into the aqueous environment (Jakubowska et al., 2007).

To summarize this section, it should be emphasized that rhamnolipids must be used in high concentrations for metal leaching processes due to their sorption in soil. Determining the optimum concentration of rhamnolipids is essential to achieve the most effective heavy metal complexation. Notably, when the concentration of rhamnolipids is too low, there is no possibility for effective heavy metal complexation. Most rhamnolipids will simply be more or less bound to the soil matrix. The significance as well as extend of rhamnolipids sorption in soil was evaluated in details by (Parus et al., 2023). Moreover, an important factor in achieving an optimal concentration of rhamnolipids in the soil is the composition and type of soil used. Some studies indicate that increased amounts of clay, soil

organic matter and aluminium, iron and manganese hydroxides increase the adsorption of rhamnolipids in the soil, which may limit the effectiveness of heavy metal complexation (Ochoa-Loza et al., 2007; Wen et al., 2010). Therefore, it should be taken into account that the CMC value in soil will be an order of magnitude higher than in water due to sorption in the soil matrix (Parus et al., 2023). It is also important not to use excessive amounts of rhamnolipids in soil systems. This ensures lower process costs and reduces the risk of potential negative effects, such as toxicity to plants or microorganisms.

A major disadvantage is that, although rhamnolipids show the ability to complex metals, they do not have the selectivity of commercial chelating agents (Table 3). Consequently, they can be used to leach metals that are significantly different in terms of chemical properties from other metals present in a selected soil. In other cases, the application of rhamnolipids will be associated with the necessity to separate the mixture of metals leached from the soil, which corresponds to higher costs.

Table 3. Conditional stability constant for heavy metals with rhamnolipids and synthetic chelating agents

Conditional stability constant						
Chelating Agent	Fe	Cu	Zn	Pb	Cd	Reference
Rhamnolipids	5.2	9.3	5.6	8.6	6.9	(Ochoa-Loza et al., 2001)
EDTA	14.0-14.3	14.5-18.8	12.0-16.5	12.0-18.0	12.0-16.5	(Broekaert, 2015)
NTA	8.8-15.8	11.5-13.2	10.1-10.6	10.6-12.4	8.6-10.1	(Anderegg, 1982)
DTPA	10.7-16.0	21.6	13.4-18.6	12.8-18.9	12.8-19.3	(Jelecevic et al., 2019)
IDA	9.8-19.3	10.6-16.3	7.0-12.4	7.3-12.1	5.5-10.0	(Anderegg et al., 2005)
MIDA	6.7-12.0	10.9-17.9	7.5-14.1	7.9-12.1	6.4-11.8	(Anderegg et al., 2005)
TTHA	7.5	8.0-13.5	8.1-18.1	6.1-18.0	8.2-18.7	(Anderegg et al., 2005)

EDTA- Ethylenediaminetetraacetic acid, NTA- Nitritotriacetic acid, DTPA- diethylenetriaminepentaacetic acid, IDA- Iminodiacetic acid, MIDA - 2,2'-(Methylimino)diacetic acid, TTHA - Triethylenetetramine-N,N,N',N'',N''',N''''-hexaacetic acid

### 3.3. Phytoextraction of heavy metals with rhamnolipids

Due to the lack of statistical effects of PS on soil washing and leaching processes, we decided to carry out phytoextraction tests without PS added to the soil.

The second experimental part was focused on the impact of rhamnolipids on the phytoextraction efficiency of the studied metal ions. This aspect was investigated since numerous scientific reports indicated that the rhamnolipids may play a crucial role in the phytoextraction of heavy metals from the

soil. Some of these studies were summarized in a recent review by (Parus et al., 2023). The most significant aspect of this experiment was associated with inconsistent results in terms of the applied dose of rhamnolipids vs. the ability of the plants to remove metals. Metals are expected to be transported by rhamnolipids applied at concentrations that prevent free movement of the biosurfactant through the soil matrix due to sorption. There is also a lack of consistency with respect to the selectivity of rhamnolipids to all metal ions present in contaminated soil. Consequently, very little is known about the actual effects of rhamnolipids on phytoextraction processes of metals in actual, non-model soil systems. Taking into account the results obtained during the soil washing experiment, it was decided to investigate whether rhamnolipids are suitable for phytoextraction of heavy metals from soil from the vicinity of a copper smelter. Since Fe was the dominant metal in the analysed soil and heavy metals were present in concentrations an order of magnitude lower, it was hypothesized that maize (*Zea mays* L.), which has previously been used by many researchers (Abedi et al., 2022; Aladesanmi et al., 2019; Ruiz-Huerta et al., 2022; Wajid et al., 2020), will preferentially accumulate Fe.

The content of the analysed metals in the roots, shoots and soil determined at the end of the experiment indicated that maize (*Zea mays* L.), in the presence of rhamnolipids applied at a concentration equal to  $1.5 \times \text{CMC}_{\text{soil}}$ , successfully accumulated more Zn, Cu, Pb. A further increase in the concentration of rhamnolipids to  $2 \times \text{CMC}_{\text{soil}}$  resulted in a more efficient uptake of Zn, Cu, Pb. These metals were primarily accumulated in the roots, and only a small portion was transported to the shoots. Cd remained firmly bound to the soil matrix and no uptake of this metal by the roots and shoots was observed. All determined parameters are presented in the Table S3 (Please see ESI for more information).

Furthermore, it was observed that an increased concentration of rhamnolipids resulted in increased values of the bioconcentration factor (BCF), the translocation factor (TF) and the enrichment factor (EF), but a significant change occurred only occasionally. The high BCF values (in the range of 1.2-1.85) for Fe indicate that Fe dominated among other metals in terms of the uptake into the roots and thus, this may limit the access of other metals to the maize (*Zea mays* L.) roots. The zero values of all coefficients indicate that maize (*Zea mays* L.) was unable to accumulate Cd. Both Cu and Pb were stabilized in the soil and roots of the plant, which is similar to the trend observed by (Olu, 2013).

Interestingly, only Zn was accumulated in greater amounts in the shoots than in the roots ( $\text{TF} > 1$ ) for the experiment with rhamnolipid addition. The compiled parameters indicate that maize (*Zea mays* L.) is not an optimal plant for phytoextraction of metals, since the content of metals transferred from the soil to the stem and leaves. In consequence, the vast majority of metals remained in the roots and soil. Such partitioning of metals means that practical and inexpensive methods of shearing the above ground part of the plant with extracted heavy metals will not be an efficient strategy for removing metals from polluted soil.

Most importantly, Fe was preferentially transported to the roots and shoots due to the lack of selectivity of rhamnolipids towards heavy metal ions (Figure 2). The biosurfactant was two orders of magnitude more effective against Fe than against the heavy metals of interest. As such, the second hypothesis regarding limited applicability of rhamnolipids during phytoextraction of heavy metal-polluted soil was confirmed.

**Figure 2.** Mean values of Zn, Cu, Pb and Fe content in soil, roots and shoots of maize (*Zea mays* L.). Note that the scale for Fe is 2 times larger than for the other metals. (different letters indicate statistical difference)

### 3.4. Phytotoxic effects towards maize after phytoextraction

In the last experimental section, the phytotoxic effects of rhamnolipids-assisted phytoextraction were evaluated. It was observed that the application of rhamnolipids, even at the lowest concentration ( $CMC_{soil}$ ), caused a noticeable and negative effect on maize (*Zea mays* L.) growth, which was observed by examining the lower shoot weight (Table 4). This toxic effect of rhamnolipids increased when they were introduced at higher concentrations, reaching approx. 50% decrease of shoot mass and 55% decrease of root mass at  $2 \times CMC_{soil}$ .

**Table 4.** Average dry weight of maize (*Zea mays* L.) shoots and roots.

Control		$CMC_{soil}$		$1.5 \times CMC_{soil}$		$2 \times CMC_{soil}$	
dry weight of shoot [g]	dry weight of root [g]	dry weight of shoot [g]	dry weight of root [g]	dry weight of shoot [g]	dry weight of root [g]	dry weight of shoot [g]	dry weight of root [g]
$1.87 \pm 0.32$	$1.55 \pm 0.46$	$1.37 \pm 0.22$	$1.25 \pm 0.31$	$1.16 \pm 0.28$	$1.03 \pm 0.23$	$0.92 \pm 0.19$	$0.85 \pm 0.17$

The negative impact of rhamnolipids was also confirmed by the analysis of obtained chlorophyll fluorescence parameters for maize (*Zea mays* L.) and the amount of chlorophyll produced (Table 5). The performance of the photosystem was lower by 25% even at the  $CMC_{soil}$  concentration of rhamnolipids. All fluorescence parameters (initial, maximum and variable fluorescence, photosystem performance and electron transport efficiency) also deteriorated after the addition of biosurfactants. Notably, this effect increased with the increase of rhamnolipid content. At the highest dose, the analysed parameters were reduced by 20-40%. This clearly indicates the phytotoxic effect of rhamnolipids applied at high concentrations ( $1.5 \times CMC_{soil}$  and  $2 \times CMC_{soil}$ ).



**Table 5.** Average values of obtained chlorophyll fluorescence parameters and amount of chlorophylls in leaves for maize (*Zea mays* L.).

Sample	Initial fluorescence ( $F_0$ )	Maximum fluorescence ( $F_m$ )	Variable fluorescence ( $F_v/F_m$ )	Photosystem performance (Y)	Electron transport efficiency (ETR)	Average amount of chlorophylls [mg/g]
Control	$255 \pm 0.47$	$1084 \pm 11.6$	$0.76 \pm 0.002$	$0.21 \pm 0.001$	$35.97 \pm 0.24$	$10.24 \pm 0.98$
$1 \times \text{CMC}_{\text{soil}}$	$234 \pm 3.3$	$1036 \pm 8.5$	$0.77 \pm 0.001$	$0.18 \pm 0.001$	$28.95 \pm 0.73$	$9.38 \pm 0.20$
$1.5 \times \text{CMC}_{\text{soil}}$	$201 \pm 2.1$	$856 \pm 9.4$	$0.77 \pm 0.001$	$0.15 \pm 0.01$	$25.34 \pm 0.72$	$8.88 \pm 0.07$
$2 \times \text{CMC}_{\text{soil}}$	$181 \pm 3.3$	$825 \pm 5.7$	$0.78 \pm 0.005$	$0.14 \pm 0.01$	$18.43 \pm 0.24$	$8.05 \pm 0.30$

The results clearly indicate that rhamnolipids used in higher concentrations can have negative effects on plants. This is due to three overlapping issues. Firstly, rhamnolipids are surfactants, and this type of compound, which combines the characteristics of a hydrophilic and hydrophobic molecule, is known to interact with cell membranes (Crouzet et al., 2020; Herzog et al., 2020). Moreover, rhamnolipids are anionic surfactants and for this reason, they behave like other surfactant compounds undergoing sorption, desorption, etc. Despite the fact that many authors cite their biological origin as a guarantee of their ecological nature (Akbari et al., 2018; Shao et al., 2017), this is quite difficult to understand, especially if such information is not supported by data. The second aspect, that promotes toxicity, is the need to use rhamnolipids in concentrations that exceed their sorption in the soil, which further increases the possibility of direct interaction with plant tissues. The third, and extremely important, factor is the mobilization of heavy metal ions, which through complexes can penetrate more easily into the interior of plants. Through this, ions interact with bio-molecules, causing oxidative stress, cellular damage, etc. As a result of these interactions, plants slow down their growth, reduce their biomass gain, and under extreme conditions may even die (Marecik et al., 2012). Therefore, rhamnolipids are transport agents that increase the availability and thus toxicity of heavy metals in soils, with even rhamnolipids already showing some toxic effects.

Our results raise more questions and issues. First, it is necessary to carefully evaluate the potential of rhamnolipids to determine whether their application is economically- and environmentally-effective in a life cycle perspective (Owsianiak et al., 2013). Second, it is unknown how rhamnolipids perform in soils of different type and properties (Yong and Phadungchewit, 1993). Third, the performance of rhamnolipids in surfactant-mediated phytoremediation is expected to depend on solid-phase reactivity of the metals in the soil, which may be different for emission sources other than smelters, and furthermore it may be influenced by aging and weathering mechanisms which are active in the soil (Owsianiak et al., 2015). Nevertheless, the results obtained can certainly be useful for anyone working

with biosurfactants, planning new experiments, or for interpreting the obtained data. Studies using aged polymers are also worthwhile, as their presence in the soil should likely reduce metal availability.

#### 4. CONCLUSIONS

The results presented in this study suggest that there may be significant limitations to the practical application of rhamnolipid-assisted soil washing and phytoextraction of heavy metal ions in non-model systems. For example, although rhamnolipids show the ability to complex metals, they do not exhibit preferential selectivity to heavy metals, which are the target contaminants in environmental clean-up attempts. Therefore, the use of this biosurfactant will require separation of the mixture of metals leached from the soil, which is not economically sound. Furthermore, the phytotoxic effects of rhamnolipids used at such high concentrations must be taken into account, as they could potentially limit the effective use of selected plants for phytoextraction. In addition, high concentrations should be used due to the sorption of the biosurfactant in the soil.

The effect of microplastic addition on soil leaching and leaching tests was statistically insignificant. In addition, experiments with PS microplastic showed negligible sorption of heavy metals on the PS surface. This can be explained by the small surface area of the polymer and the lack of a surface functional group typical of virgin, unaged polymers.

Hence, it is necessary to carefully evaluate the commercial potential of rhamnolipids and to determine whether their application is feasible for bioremediation of heavy metal-contaminated soils.

The results obtained can certainly be useful for anyone working with biosurfactants, planning new experiments, or for interpreting the obtained data. Yet, additional life-cycle-based analyses are needed to assess the overall environmental and economic effectiveness of rhamnolipid-assisted remediation as a management strategy for contaminated soils. Studies using aged polymers are also worthwhile, as their presence in the soil should likely reduce metal availability.

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