Metal Removal from Contaminated Soils Trough Bioleaching with Oxidizing Bacteria and Rhamnolipid Biosurfactants

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METAL REMOVAL FROM CONTAMINATED SOILS TROUGH BIOLEACHING WITH OXIDIZING BACTERIA AND RHAMNOLIPID BIOSURFACTANTS

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ABSTRACT

The use of surfactants as a method for solubilisation and removal of heavy metal contamination from soil has been reported before. Biosurfactants produced by some microorganisms are able to modify the surface of various metals and aggregate on interphases favouring metal separation process from contaminated environments. We evaluated the feasibility of enhancing the removal of metal ions from a mineral waste/contaminated soils using alternate cycles of treatment with rhamnolipid biosurfactants and bioleaching with amixed bacterial culture of *Acidithiobacillus thiooxidans* and *Acidithiobacillus ferrooxidans*. Bioleaching alone removed 50% Zn and 19% Fe. When rhamnolipids was used at low concentration (0.4 mg/mL) 11% Fe and 25% Zn were removed while at 1 mg/mL 19% Fe and 52% Zn removal were achieved. When using a cyclic treatment combining bioleaching and biosurfactants, metal removal reached up to 36% for Fe and 63% to 70% for Zn.

Key words: Metal removal, bioleaching, soil washing, rhamnolipids, *Pseudomonas aeruginosa*

INTRODUCTION

Waste from metal ore mining processes is one of the largest streams with potential environmental impact which has left a historical legacy of contaminated land. High metal content as well as the associated toxicity represents a great risk for ecosystems and human health. This kind of residue often contains sulphide minerals such as pyrite (FeS_2) which oxidizes in the presence of air and water producing iron and sulphate in solution and which ultimately leads to acidic conditions in the waste stream medium and acid mine drainage and associated environmental contamination affecting both surface and ground water (Akcil and Koldas, 2006). In addition, mining residues tend to modify contaminated soils changing their intrinsic properties producing several effects, such as: imbalance of essential nutrient content, disruption of biogeochemical cycles, low exchange capacity or low water retention (Puga et al., 2006). The presence of metals such as arsenic, cadmium, zinc, copper, chromium, manganese, mercury, lead and even cyanide species have confirmed detrimental effects on the chemical quality of soils (Alloway, 1995; Pinochet et al., 2002).

Some technologies used in the remediation of metal contaminated soils are based on physical separation (gravity concentration, magnetic separation or electrostatic separation) or chemical extraction (acid or chelate extraction). The use of microorganisms for metal extraction, known as bioleaching, has been investigated for a long time. The mechanisms involved describe different redox reactions mediated by enzymatic processes. Different strains of *Acidithiobacillus* have been reported for their ability to leach base metals such as iron, copper and zinc (Rodríguez et al., 2003).

Extraction of metals by surfactants thought solubilisation has been known and previously applied with effective results (Stalikas, 2002). Both cationic and anionic surfactants have been used as washing or flushing agents to extract metals from aqueous solutions by counterion binding (Mulligan and Wang, 2006; Wang et al., 2004) without involving any direct or indirect mechanism of bioleaching.

Rhamnolipids are surfactants of biological origin commonly produced by strains of the genus *Pseudomonas.* Their amphiphilic structure allows them to partition on interphases increase the bioavailability and or mobilisation of hydrophobic substrates (Banat et al. 2010). Based on their surface activity, rhamnolipids can be used in many industries as emulsifiers, foaming or dispersing agents (Marchant and Banat, 2012a,b). At present special attention are directed towards environmental applications in which their properties could be useful for the development of physicochemical and biological remediation technologies (Pacwa-Plociniczak et al., 2011). These compounds may form complexes with metals thus facilitating their desorption from contaminated soils particles and leading to a better bioavailability in the aqueous phase. This approach would allow the subsequent extraction and recovery of these metals improving the environmental conditions on the site. Biosurfactants' ability to reduce the interfacial tension allows direct contact with the adsorbed metals. Consequently, cationic metals could be removed from soils by flushing through with anionic biosurfactants such as rhamnolipids. Comparative studies using different types of surfactants have demonstrated the effectiveness of rhamnolipids in the removal of Zn, Cu, Pb and Cd from a soil exposed to metal deposition (Slizovskiy et al., 2011).

Most studies undertaken so far have focused on the use of surfactants/biosurfactant only as a mechanism for dissolving metals through washing system, or either the use of bacteria using the bioleaching process. In this study we evaluate the feasibility to enhance metal removal from a mineral waste using an alternate cycle of two different treatments: with a biosurfactant obtained from a *Pseudomonas aeruginosa* strain and with a bioleaching process using a mixed culture of bacteria belonging to the genus *Acidithiobacillus*. Our model makes use of surfactant as a facilitator for the separation of metal from the contaminated soil, opening a way for the bioleaching mechanism, using the synergy between two processes demonstrating the effectiveness of the model.

MATERIALS AND METHODS

Sampling site

The samples for the microbial study were collected from an old closed mine site named "Brunita" near Llano del Beal (Sierra Cartagena-La Unión, Murcia, Spain) and formerly used as a source of Pb–Zn ores. The soil samples were dried at room temperature and subsequently the material was sieved through a 1mm sieve and particles >1mm were discarded. The small particles (<1 mm) met the requirements for assessment of soil by sequential extraction technique (Brady et al., 1999) and were characterized physically, chemically and mineralogically to be used in the different studies.

Physical characterization of the contaminated soil

The contaminated soil (CS) were homogenized and divided into portions of about 2 kg and kept in plastic bags in a refrigerator at −4°C. Samples preparation for the various assays was carried out according to ASTM C702. The particle size distribution was determined using the *Tyler*

sieve series mesh size of 1000, 750, 600, 430, 250 and 100μm. The bulk density was determined by measuring the volume occupied by a given mass of dry soil in a container of known dimensions. While specific density was obtained by calculating the specific volume occupied by a known mass of ground in a known density liquid (deionized water) using a pycnometer.

Chemical characterization of the contaminated soil

Soil pH, redox potential, and the concentration of potentially contaminating metals (Fe, Cu, Zn, Pb) were carried out. The pH and redox potential of the soil was determined with two contact media: deionized water of pH 5.6 and nutrient medium 0K of pH 1.8. For this, 10g of soil and 90mL of liquid media were placed in a 250mL Erlenmeyer flask with constant stirring at 150rpm. pH and redox potential of the suspensionwere measured after 1, 22 and 120h, with a Ag/AgCl and a platinum electrode, respectively, in a pH meter Crison Basic model 20 (Rayment et al., 2006). In the same way 10g of soil and 90mL of deionized water were placed together and pH was determined. The availability of metals from different soil was determined by sequential extraction according to Brady et al. (1999) (Figure 1).

 Mineralogical characterization of the contaminated soil

Mineralogical characterization was carried out by powder X-ray diffraction (XRD) on a Phillips X'pert-MPD system using Cu-Kα=1.5406 $\mathring{A}$. The scanning range was from 10° to 70° 2θ with an angular interval of 0.05° and 4s counting time. The crystalline phases were identified using standard cards from the International Centre for Diffraction Data (ICDD, Newtown Square, Pennsylvania) Powder Diffraction File database.

Bacterial cultures

A mesophilic mixed culture of Fe- and S-oxidizing microorganisms (*Acidithiobacillus thiooxidans* and *Acidithiobacillus ferrooxidans*) was provided by the Department of Materials Science and Metallurgical Engineering, Complutense University of Madrid, Spain. The bacterial culture was grown at 30°C on 0K medium (3g/L $(NH_4)_2SO_4$, 0.1g/L K_2HPO_4, 0.5g/L $MgSO_4 \cdot 7H_2O$, 0.1g/L KCl, and 0.013g/L $Ca(NO_3)_2 \cdot 4H_2O$), in a 250 mL flask at 200 rpm, pH 1.7.

Rhamnolipids (Rhl's) were produced from a strain of *Pseudomonas aeruginosa* CVCM 411 provided by the Venezuelan Central Collection of Microorganisms. The culture was grown in 250mL flasks, at 200 rpm and 37°C on PPGAS medium (0.02M NH_4Cl, 0.02M KCl, 0.12M tris-HCl, 1% peptone and $MgSO_4 \cdot 7H_2O$ 0.01%) at pH=7.4, for 72h. Rhamnolipids were collected from solution following the method proposed by Dèziel et al. (1999) and then stored at 4°C until use.

Microcosm tests

The microcosms were prepared in 250mL Erlenmeyer flasks containing 10g of sterilized contaminated soil, 90mL of 0K medium (pH 1.7) and 10mL of a cell suspension which was withdrawn from stocks of mixed cultures of Fe- and S-oxidizing bacteria in exponential growth phase ($>10^8$ cell/mL). Tests were carried out in triplicate (with yield differences lower than 5%), in an incubator at 30ºC and 150 rpm (Rodríguez et al., 2003). A sample of 2mL was removed from solution after 7, 14, 21, 28, 35, 42 days and analysed for metal content (Fe and Zn).. The solid residues were dried and analysed following a sequential extraction procedure. Control test

was carried out in the presence of water without the mixed culture of Fe- and S-oxidizing bacteria.

Washing tests

The contaminated soils were washed with rhamnolipids simulating spot removal techniques. Biosurfactants solutions (original stock 0.1% v/v) were placed in a 500mL flask at two different concentrations (0.4 and 1 mg/mL). Both tests contained 10g of sterelized contaminated soil in 0K medium at pH 8 for a final volume of 100mL. Washing tests were run according to the protocol described by Mulligan et al. (2001) with slight modifications (30 h, 150 rpm and 25°C). A sample of 2mL was removed after 0.25, 1, 3 and 30h to determine the percentage of iron and zinc leached. The solid residues were dried and analysed following a sequential extraction procedure.

Additional washing tests were carried out with the highest concentration of rhamnolipids (1 mg/mL) in 0K medium at pH 8 for 30h. After that time, the contents were centrifuged at 10,000g for 15min and the supernatant was used to determine the concentration of metals removed. The residue was then collected, dried and placed in contact with a mixed bacterial culture for 21 days according to the experimental procedure described in the microcosm tests.

Cyclic treatment

This experimentation consisted of alternate treatment cycles of washing (Rhl's), microcosm (mixed culture), washing (Rhl's), to complete a period of time of 25 days (Figure 2).

The first cycle was started under the experimental conditions referred in the washing tests. After 24h, the entire volume was centrifuged at 10,000g for 15min and the supernatant used to determine the concentration of metals leached. The solid residue was dried and placed in contact

with the mixed bacterial culture suspended in 0K medium (100mL) under the experimental conditions described in the microcosm tests.

After 7 days of incubation, the total volume was centrifuged at 10,000g following the same procedure as before. The concentration of metals removed was evaluated in the supernatant and the residue of the bacterial treatment was dried and used in washing tests. Again, after 24h, the supernatant obtained by centrifugation was analysed for metal content. This alternate treatment was repeated for a period of 25 days.

RESULTS AND DISCUSSION

Characterization of Contaminated Soil (CS)

Physical characterization

One of the soil primary functions is the regulation of the water flow in the hydrological system. Two parameters that determine the degree of permeability of the soil are its particle size and its density. The particle size distribution of the contaminated soil under study is depicted in Figure 3 and shows that it mainly contains fine particles, 80% by weight having a size less than 250 microns. Such size distribution suggests the presence of weathering products coming from mining-metallurgical waste with a fine particle size and as suggested later containing jarosite as the main component identified by XRD. Consequently, the lower permeability of the CS would result in an increase of water retention, a key element in the biogeochemical transformations of metal sulphides. Soil density can be obtained under two criteria as specific or bulk density. Accordingly, the specific density of a soil tends to be greater than its bulk density (Brady and

Weil, 2008). In this regard, the higher specific gravity of the contaminated soil (Table 1) may be attributed to the presence of mining residues, most likely the metallic sulphides.

Chemical Characterization

CS was characterized from representative samples that were analysed for pH and redox potential in contact with a liquid medium and the metal content. Redox potential and pH are two variables that govern the retention and/or mobilization of metals in soils (Plant and Raiswell, 1983; Alloway and Ayres, 1993). Measurements in pH showed a significant drop in deionized water, from 6.11 to 2.41, while in in the medium 0K slight increase from 1.85 to 2.37. This is a consequence of the strong acidic composition of the CS, which is reflected in the trend of a single pH value after 5 days of contact between the CS and both liquid media. The high acidity of CS would result in the dissolution of the mineral phases with buffering capacity, such as carbonates and hydroxides. Hence, this would have a detrimental effect on the cation exchange capacity of this soil (Konhauser, 2007; Martín et al., 2007; Brady and Weil, 2008).

The redox potential of the CS in deionized water and 0K medium suggests that this soil has oxidizing properties that, similar to pH, promotes metal mobilization (Sharpe, 1993). Therefore, the values of pH and redox potential recorded during these trials are indicatives that the contaminated soil could promote the growth of acidophilic and autotrophic bacteria, such as *Acidithiobacillus spp.*, which play a key role in the mobilization of toxic metals (Holt et al., 1994; Ballester et al., 2000).

Metal content and bioavailability in CS

The determination of metal content in CS by sequential extraction was carried out in triplicate on representative samples. A widely used parameter for the declaration of contamination of soils is metal concentration. The choice of metallic elements to be evaluated in this study was conducted on the basis of their relative abundance in the sample and in accordance with data previously reported (García et al., 2007; Robles -Arenas et al., 2006). The total concentration of metals (Fe, Cu, Zn and Pb) in the CS is shown in Table 2. The values for Pb and Zn were 7 and 5 times greater than the limit values set by the CMJA (Andalusian Ministry of the Environment) for Pb and Zn, respectively.

Mineralogical characterization

In most soils, metals are present as carbonates, oxides, sulphides or salts. While the dominant minerals of each metal can vary from one soil to another, neither of these minerals can self-regulate the metal concentration in the aqueous phase of the soil (Brady and Weil, 2008). According to the characteristics of mining-metallurgical activities developed in the sampling area of the CS in the Sierra Cartagena-La Union, it is expected that soil pollution is associated with sulphidic material present in its mineral composition (García et al., 2007; Robles-Arenas et al., 2006). In this sense, the determination of the principal mineral phases in the CS was performed on powdered samples of the original soil. The XRD pattern for the contaminated soil showed oxidized phases (quartz, silicates and sulphates -gypsum and plumbojarosite-) as major constituents and to a lesser extent iron sulphides (pyrite). However, the significant presence of zinc recorded by sequential extraction (Table 2) was not confirmed in XRD analysis of the raw

CS. This could be attributed to zinc be forming an amorphous compound or adsorption to the soil.

Microcosm tests

The results obtained in the microcosm at 30°C are shown in Figure 4. Both iron and zinc in solution increased over time. The leaching of metal sulphides is usually accompanied by an increase in metal concentration in the leachate (Bosecker, 1997). It is well known that the dissolution of pyrite by ferric sulphate leads to ferrous sulphate as observed in reaction [1]. In turn, Fe-oxidizing microorganisms are able to catalyse the oxidation of ferrous into ferric sulphate, either in its ionic (reaction [2]) or molecular form (reaction [3]) (Jensen and Webb, 1995; Acevedo and Gentina, 2005). Pyrite is not dissolved in acidic solutions but attacked exclusively by an oxidizing agent such as Fe^{3+}. The presence of Fe-oxidizing bacteria would therefore be necessary to regenerate the oxidizing agent required for leaching:

$$FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 16H^+ + 2SO_4^{2-} \tag{1}$$

$$2Fe^{2+} + \tfrac{1}{2}O_2 + 2H^+ \xrightarrow{\ Bacteria\ } 2Fe^{3+} + H_2O \tag{2}$$

$$FeSO_4 + H_2SO_4 + \tfrac{1}{2}O_2 \xrightarrow{\ Bacteria\ } Fe_2(SO_4)_3 + H_2O \tag{3}$$
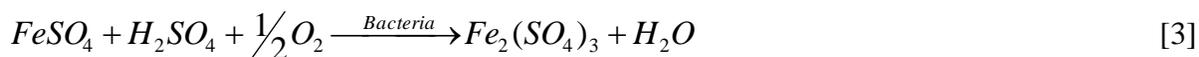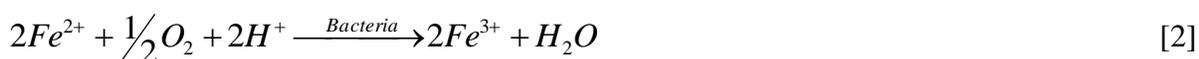
On the other hand, the leaching rate for zinc is much faster than that for iron (Figure 4) and also resulting in higher final metal extractions (50% Zn vs. 19% Fe). This is likely due to zinc sulphides being more soluble in acidic conditions than pyrite and its ability to dissolved by oxidizing agents such as ferric iron(Acevedo and Gentina, 2005):

$$ZnS + Fe_2(SO_4)_3 \rightarrow ZnSO_4 + S^0 + 2FeSO_4 \qquad\qquad [4]$$

$$ZnS + 2H^+ \rightarrow Zn^{2+} + H_2S \qquad\qquad [5]$$

The presence of Fe(III) in solution accounts for the pyrite dissolution, a process that generates a great amount of Fe(II) that can be oxidized by bacteria to Fe(III) (Rodríguez et al., 2003). Previous studies have shown that the bioleaching of complex metallic sulphides with mesophilic mixed cultures of the genus *Acidithiobacillus* yields high zinc extractions (around 90%), as shown in reactions [4] and [5] (Deveci et al., 2004; Wang et al., 2004).

Washing tests

Washing experiments were performed at pH 8 and at two different rhamnolipid concentrations (Figure 5). The amount of iron leached in the presence of 1 mg/mL of Rhl's was comparatively higher than with 0.4 mg/mL of the same surfactant (19% vs. 11%, respectively). In the case of zinc, a similar trend was observed after 30h of washing: 52% of Zn was dissolved in the presence of 1 mg/mL of RhL's compared with 25% of Zn with 0.4 mg/mL of Rhl's (Figure 5). These results confirm previous reports by Mulligan and Wang (2006). The residues obtained were analysed to determine the distribution of iron and zinc after washing. It was expected a greater transformation of iron from the sulphide fraction than from any other fraction, releasing Fe^{3+} as expected in reactions [1] and [2] (Jensen and Webb, 1995). Consequently, the decrease of iron in the sulphide fraction detected in the microcosms (as shown in Table 3) was attributable to the washing step mediated by the presence of rhamnolipids.

Under our experimental conditions rhamnolipids biosurfactant stimulated a preferential dissolution of iron from the sulphide fraction that affected its final distribution in different soil

fractions. Unlike iron, which showed a higher concentration in the sulphide and residual phases in the contaminated soil, zinc was more concentrated in the exchangeable and sulphide phases followed by the hydroxide and carbonate phases (Table 3).

According to Table 3, the significant decrease in the exchangeable and carbonate phases could be attributable to a higher bioavailability of toxic metals from these fractions. Metals in the exchangeable fraction are weakly adsorbed by electrostatic interactions and are easily available in natural environments (Li et al., 2001). On the other hand, mobilization of metals from the carbonate phase is favoured by pH changes caused by different environmental agents (Gleyzes et al., 2002). Finally, the availability of zinc in the sulphide fraction may be affected by the formation of a diffusive layer of elemental sulphur on the sulphide surface (Rodríguez et al., 2003).

The microcosm tests to which bacterial cultures were added showed that iron and zinc could be leached from the soil up to 19% and 50%, respectively. Similar results (19% Fe and 52% Zn) were obtained in the washing tests but only after 24 h and pH 8.0. In the latter case, after 24h of washing with 1 mg/mL rhamnolipids followed by 21 days of contact with an oxidizing bacterial mixed culture, a 25% of iron recovery was achieved (Table 4). For tests that started with the mixed culture followed by a washing step, a final iron extraction of 22% was obtained (Table 5). In both cases, the increase in the iron recovery is an indication that the use of oxidizing bacteria in combination with rhamnolipids improves the removal of iron from the contaminated soil.

Cyclic treatment

The cyclic treatment tests were designed to increase performance of the removal of heavy metals from the contaminated soil. When using alternate cycles of bioleaching and washing tests and a frequency of 6 days between Fe- and S-oxidizing bacteria and rhamnolipids, the percentage of iron in the aqueous phase increased up to 36% (Table 6). For zinc, the improvement was noticeable reaching a final zinc recovery of 70%. The increase in the removal of both metals is attributable to a joint action of bacterial activity from the mixed culture and the washing effect of rhamnolipids. When this effect is repeated several times, under the right conditions, it is possible to increase metal removal from contaminated soils and promote a phase change of each metal after both treatments (bioleaching and washing).

The results obtained indicate that it is possible to achieve significant removal of metals such as iron and zinc from a mining waste using a mixed population of *Acidithiobacillus thiooxidans* and *Acidithiobacillus ferrooxidans.* Such removal can be enhanced by a previous washing step with rhamnolipids biosurfactants in solution at pH 8. Rhamnolipids form micelles at pH 6.8 with molecules charged negatively while their surface activities increase at higher pH 7.0 and 7.5 (Mulligan and Wang, 2006). Consequently, the effectiveness of rhamnolipids for metal separation can be greatly affected by pH change. An optimum desorption of Cd (II) and Zn(II) from quartz in the presence of rhamnolipid biosurfactants was reported to occur at pH 6.8 by Mulligan and Wang (2006).

Oxidizing bacteria participate via an indirect bioleaching mechanism through to the regeneration of the oxidizing agent (Fe^{3+}) while the role played by the rhamnolipids can be related to the complexation and micelle formation with metal ions adsorbed on mineral surfaces. During this

process, metal distribution between the different soil fractions is remarkably modified, especially the removal of zinc from the exchangeable and sulphide fractions. The ability to wash such contaminated soils to remove heavy metals may have some application in future process for removal and or recovery of such metals for contaminated environments.

CONCLUSIONS

This work demonstrates that a combined strategy may be a possible way for the treatment of heavy metal contaminated soil exposed to acid mine drainage. This is possible to achieve using a process involving washing with mixed bacterial population of *Acidithiobacillus thiooxidans* and *Acidithiobacillus ferrooxidans*, in combination with surface active molecules. The use of cycles in such washing process has to be optimized for future work in terms of energy costs, residence time and possible recovery of such metals.

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Table 1. Specific and bulk densities (ρ) for the contaminated soil.

Sample	$\rho_{specific}$ (g/cm³)	ρ_{bulk} (g/cm³)
Contaminated Soil (CS)	2.98	1.12

Table 2. Distribution of metals in the different fractions of the contaminated soil.

Soil fraction	mg Fe/Kg CS	mg Cu/Kg CS	mg Zn/Kg CS	mg Pb/Kg CS
Exchangeable	2587.09	28.34	1,538.27	347.00
Organic	7.06	10.82	6.02	25.04
Carbonates	45.98	4.00	59.44	198.71
Hydroxides	847.66	5.10	39.09	33.98
Sulphides	278,812.39	116.92	3,363.07	12,896.60
Residual	13,476.15	37.06	724.05	4,464.42

Table 3. Content of Zn and Fe in each contaminated soil fraction after 30h of washing with two different concentrations of rhamnolipids (A: 0.4 mg/mL; B: 1 mg/mL). The control test is referred as mgZn/Kg CS and mgFe/Kg CS.

Soil fraction	mg Zn/Kg CS	mg Zn A/KgCS	mg Zn B/Kg CS	mg Fe/Kg CS	mg Fe A/Kg CS	mg Fe B/Kg CS
Exchangeable	268,510	145,110	124,350	8,680	5,150	5,550
Organic	1,050	440	260	20	0.00	20
Carbonate	9,780	3,060	2,180	150	110	140
Hydroxide	6,810	1,750	870	2,840	850	700
Sulphide	587,120	296,790	156,800	935,610	754,500	642,820

Table 4. Percentage of Fe and Zn dissolved after 24h of washing with 1 mg/mL of rhamnolipids (v/v) followed by 20 days of contact with a mixed culture of *A. thiooxidans* and *A. ferrooxidans*.

Treatment	Time (days)	% Fe leached	% Zn leached
Rhl's	1	18.45	57.00
Bac	20	6.76	6.00
% Total Leachate		**25.21± 0.81**	**63± 0.35**

Rhl's = rhamnolipids solution

Bac = Bacteria culture

Table 5. Percentage of Fe and Zn dissolved after 20 days of bioleaching with a mixed culture of *A. thiooxidans* and *A. ferrooxidans* followed by 24h of washing with 1 mg/mL of rhamnolipids.

Treatment	Time (days)	% Fe leached	% Zn leached
Bac	20	15.8	45.86
Rhl's	1	6.26	3.21
% Total Leachate		**22.06± 0.42**	**49.07± 0.18**

Rhl's = rhamnolipids solution

Bac = Bacteria culture

Table 6. Percentage of Fe and Zn dissolved after an alternate cyclic test (RhL's/Bioleaching/RhL's) starting with a washing test with1 mg/mL RhL's (v/v) for 24h, then a bioleaching test with a mixed culture of *Acidithiobacillus* every 7 days.

Treatment	Time (days)	% Fe leached	% Zn leached
Rhl's	1	17.39	53.86
Bac*	7	1.19	2.93
Rhl's	1	5.07	2.00
Bac*	7	1.59	4.07
Rhl's	1	5.86	2.29
Bac*	7	1.42	3.00
Rhl's	1	3.54	1.79
% Total Leachate		**36.06± 0.268**	**69.94± 0.118**

* Treatment with bacteria performed in each mineral residue after washing.
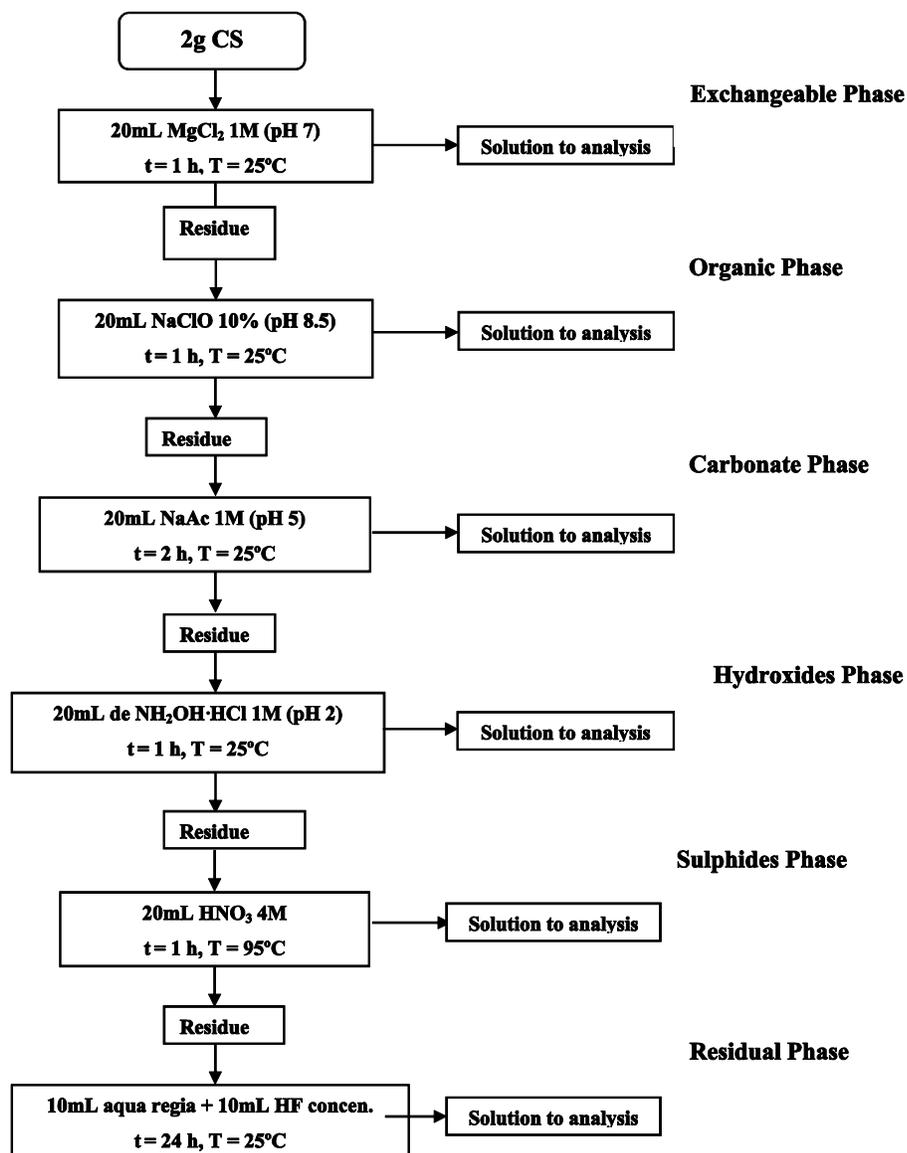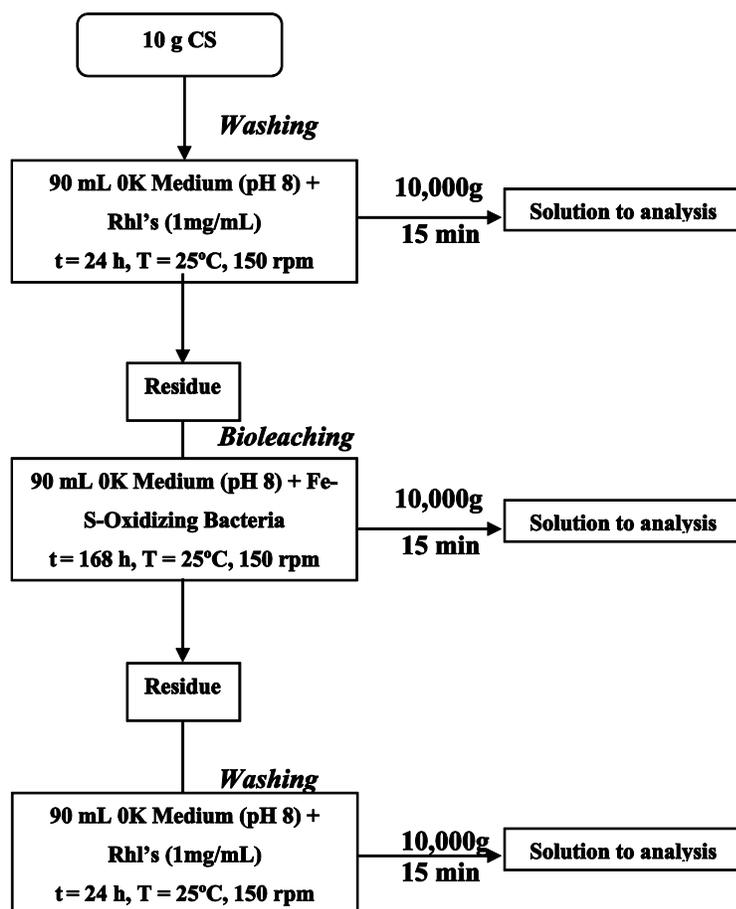
Rhl's = rhamnolipids solution

Bac = Bacteria culture

Figure 1. Stages of the sequential extraction procedure (Brady et al., 1999).

Exchangeable Phase

2g CS → 20mL MgCl$_2$ 1M (pH 7), t = 1 h, T = 25°C → Solution to analysis

Residue

Organic Phase

20mL NaClO 10% (pH 8.5), t = 1 h, T = 25°C → Solution to analysis

Residue

Carbonate Phase

20mL NaAc 1M (pH 5), t = 2 h, T = 25°C → Solution to analysis

Residue

Hydroxides Phase

20mL de NH$_2$OH·HCl 1M (pH 2), t = 1 h, T = 25°C → Solution to analysis

Residue

Sulphides Phase

20mL HNO$_3$ 4M, t = 1 h, T = 95°C → Solution to analysis

Residue

Residual Phase

10mL aqua regia + 10mL HF concen., t = 24 h, T = 25°C → Solution to analysis

24

Figure 2. Stages of the cyclic treatment (each stage was repeated for a period of 25 days).

10 g CS

Washing

90 mL 0K Medium (pH 8) + Rhl's (1mg/mL)
t = 24 h, T = 25°C, 150 rpm

10,000g
15 min → Solution to analysis

Residue

Bioleaching

90 mL 0K Medium (pH 8) + Fe-S-Oxidizing Bacteria
t = 168 h, T = 25°C, 150 rpm

10,000g
15 min → Solution to analysis

Residue

Washing

90 mL 0K Medium (pH 8) + Rhl's (1mg/mL)
t = 24 h, T = 25°C, 150 rpm
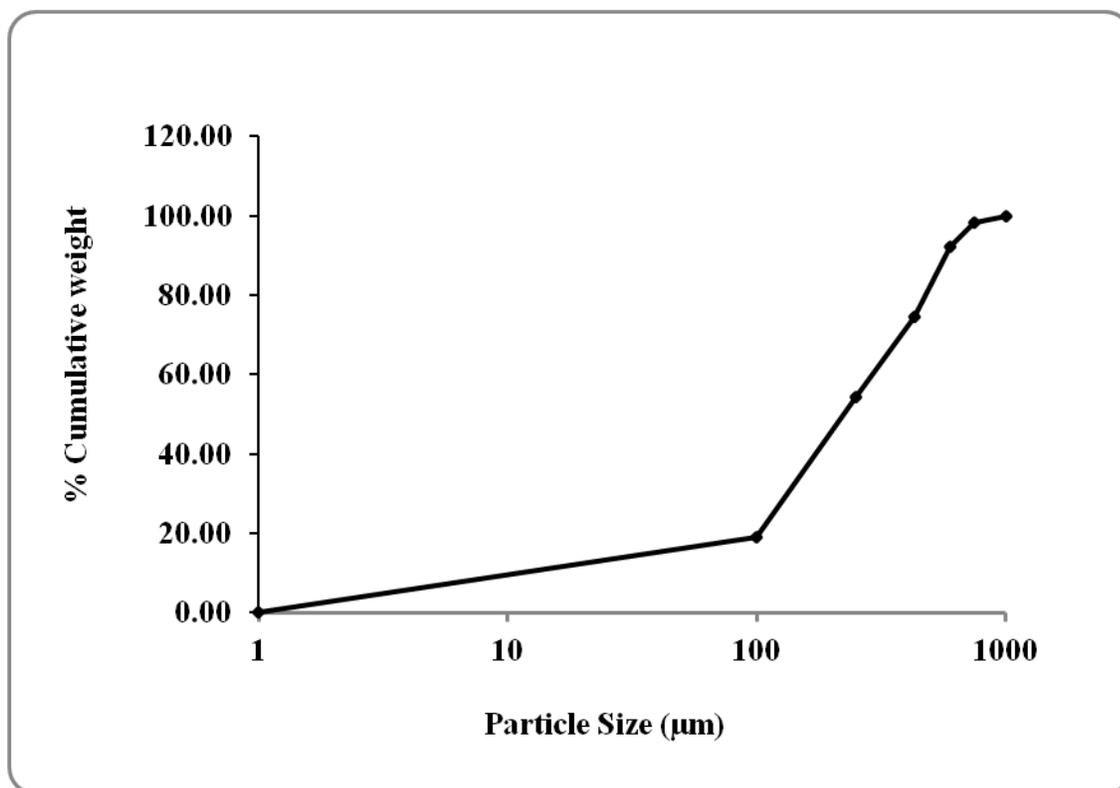
10,000g
15 min → Solution to analysis

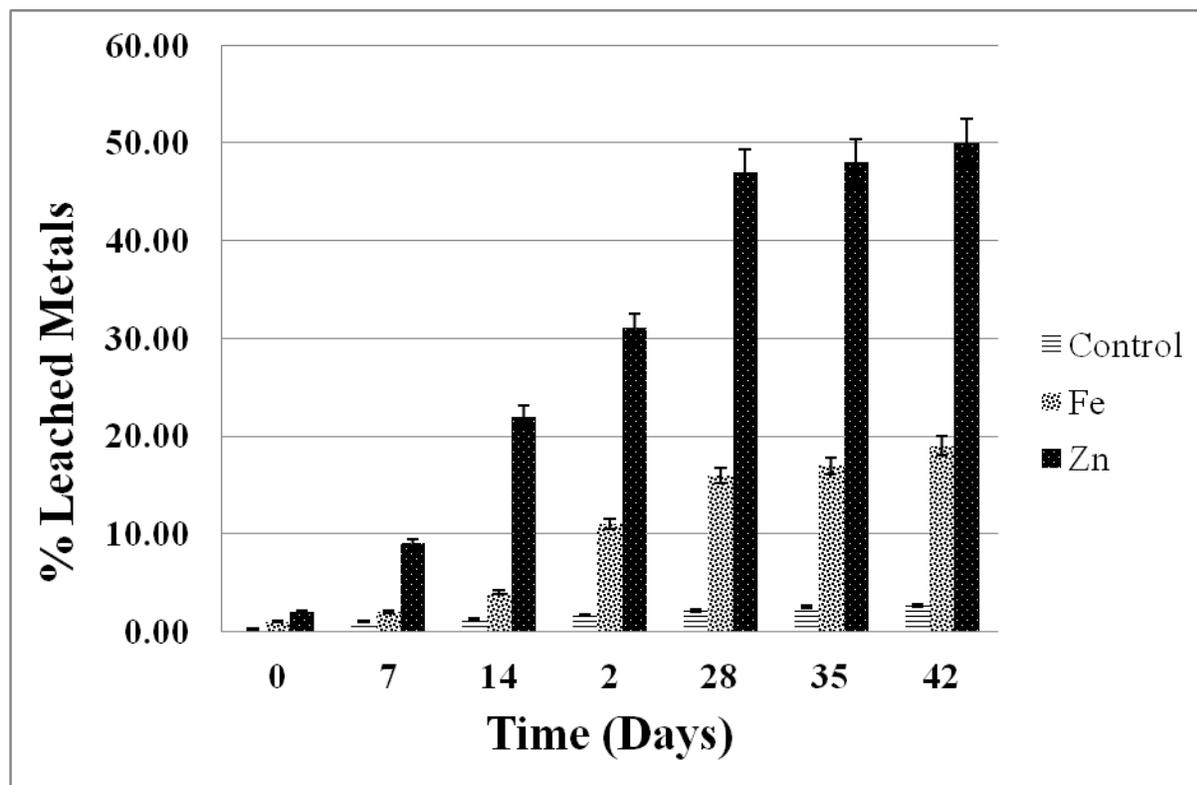**Figure 3.** Granulometric size distribution analysis of the contaminated soil.

Figure 4. Percentage of metal dissolved (Fe and Zn) in the microcosm test with a mixed culture of *Acidithiobacillus* after 42 days of bioleaching. Notice that deionized water was used as control for all the experiments.

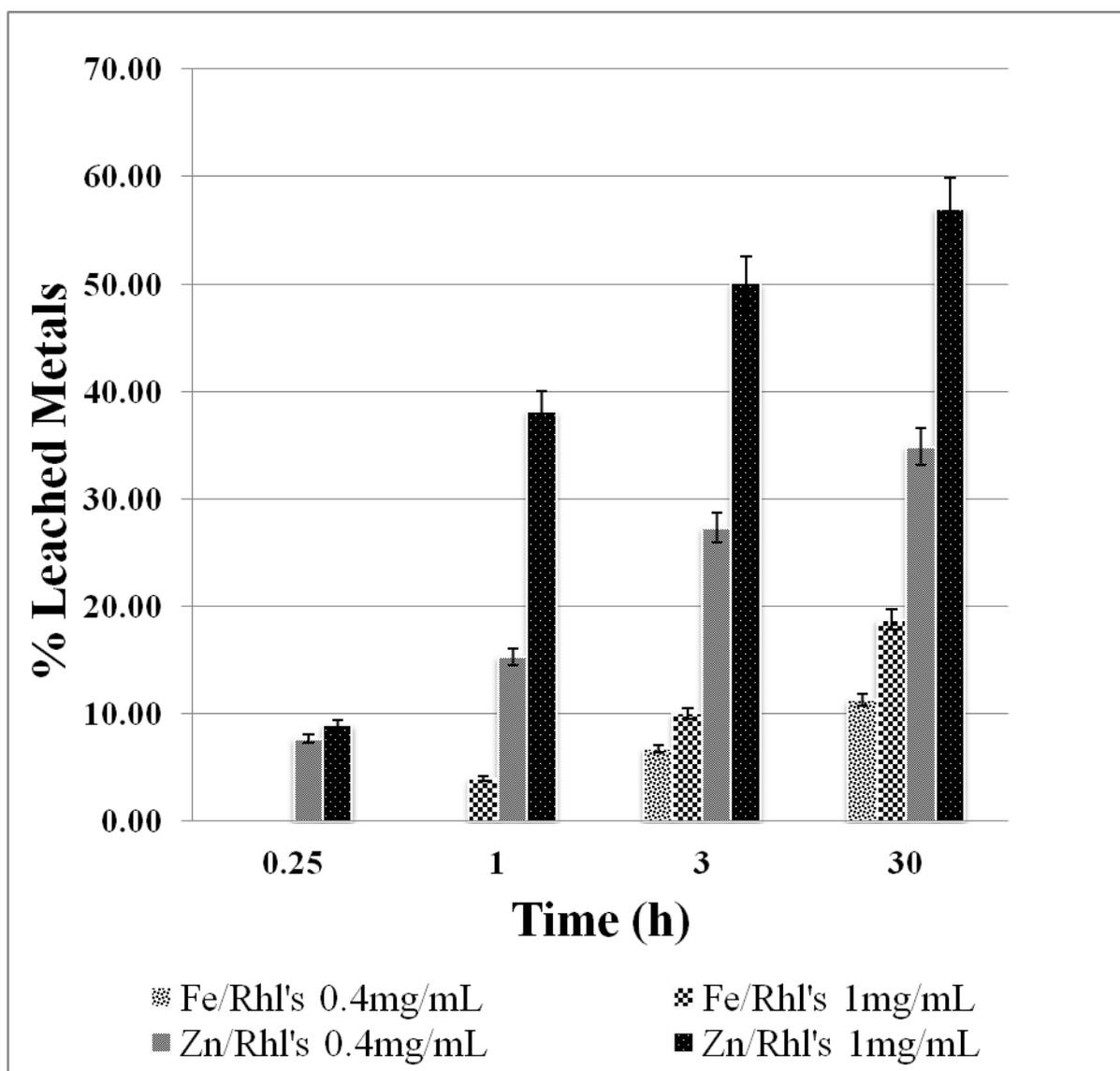**Figure 5.** Percentage of Fe and Zn dissolved in washing tests with two different concentrations of rhamnolipids (0.4 and 1 mg/L) after 30 h.