Chapter 8 Phytoremediation of heavy metals in petroleum-contaminated soil using red mangrove (*Rhizophora mangle*) and a microbial consortium

Capítulo 8 Fitorremediación de metales pesados presentes en suelo contaminado con petróleo crudo utilizando mangle rojo (*Rhizophora mangle*) y un consorcio microbiano

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Abstract

In the present work, was studied the capacity of red mangrove (Rhizophora mangle) seedlings to phytoremediator heavy metals in soils contaminated with crude oil by applying bioaugmentation through a microbial consortium made up of Serratia marcescens, Trichoderma harzianum and Rhizopus sp. Experimental treatments were prepared in triplicate with soils contaminated with 30, 60 and 100 mg/Kg of crude oil and cohorts or surveys of experimental units were carried out 0, 15, 30, 60 and 90 days, respectively. The growth of the Rhizophora mangle seedlings was evaluated, obtaining an average height of 9.69 cm and a root length average of 10.67 cm. To determine the concentration of heavy metals, the EPA 3050B digestion method was used, and for its quantification, a Thermo-Scientific atomic absorption spectrophotometer, model ICE 3000, was used. With the results obtained, the heavy metal phytoremediation percentages were calculated. Finality, the percentages of average phytoremediation of heavy metals by applying Rhizophora mangle and the microbial consortium, presented the following order: Cd (86%) > Pb (65%) > Cu (57%) > Zn (43%) > (40%).

Phytoremediation, Heavy metals, Crude oil, Red mangrove, Bioaugmentation

Resumen

En el presente trabajo, se estudió la capacidad de plántulas de mangle rojo (Rhizophora mangle) para fitorremediar metales pesados en suelos contaminados con petróleo crudo mediante la aplicación de bioaumentación a través de un consorcio microbiano conformado por *Serratia marcescens, Trichoderma harzianum y Rhizopus* sp. Los tratamientos experimentales se prepararon por triplicado con suelos contaminados con 30, 60 y 100 mg/Kg de petróleo crudo y se realizaron cohortes o muestreos de unidades experimentales a 0, 15, 30, 60 y 90 días, respectivamente. Se evaluó el crecimiento de las plántulas de Rhizophora mangle, obteniendo una altura promedio de 9.69 cm y una longitud de raíz promedio de 10.67 cm. Para determinar la concentración de metales pesados se utilizó el método de digestión EPA 3050B, y para su cuantificación se utilizó un espectrofotómetro de absorción atómica Thermo-Scientific, modelo ICE 3000. Con los resultados obtenidos se calcularon los porcentajes de fitorremediación de metales pesados. Finalmente, los porcentajes de fitorremediación promedio de metales pesados mediante Rhizophora *mangle* y el consorcio microbiano, presentaron el siguiente orden: Cd (86%) > Pb (65%) > Cu (57%) > Zn (43%) > (40 %).

Fitorremediación, Metales pesados, Petróleo crudo, Mangle rojo, Bioaumentación

1. Introduction

Currently, worldwide, energy security in oil-producing countries is still based on petroleum derivatives; therefore, it is necessary to continue research on bioremediation technologies for sites contaminated with crude oil and heavy metals; one of them is phytoremediation. Phytoremediation is an environmentally friendly and efficient technique to reduce the impact of heavy metals contained in crude oil (Barea *et al*., 2005; Shen *et al*., 2023; Singh & Pant, 2023; Tehrani & Besalatpour, 2023). This results in a feasible, economical and important method for the reduction of heavy metal contamination in the Gulf of Mexico and in the Laguna de Términos region where the red mangrove (Rhizophora mangle) predominates, which represents an important ecological alternative for phytoremediation of heavy metals present in soils contaminated with crude oil.

Bioremediation and phytoremediation are techniques that use microorganisms, plants, the interaction of plants with microorganisms, and specifically, the interaction of bacteria and fungi individually or in the form of a microbial consortium, which in turn are associated with plants (Saha *et al*., 2021; Wojtowicz *et al*., 2023). Bioremediation is an environmentally friendly technology for remediating contaminated soils, the efficacy of which requires further research as proposed in the present study. Other researchers indicate that the combined use of microorganisms or microbial remediation, plants, conditioned natural materials or even the use of nanomaterials was suggested as an effective and innovative method for remediation of soils contaminated with heavy metals (Luo *et al*., 2017; Zanganeh *et al*., 2022).

The remediation of sites contaminated with heavy metals, crude oil and other toxic pollutants; has been effective and reliable due to their ecological characteristics given that they use microbial (bioremediation) or plant (phytoremediation) processes (Moreira *et al*., 2013; Al-Solaimani *et al.*, 2022; Nayak, Bhushan & Wilson. 2022; Cheng *et al.*, 2023). Bioremediation can be performed with ex situ or in situ techniques; its application will depend on 1 the characteristics of the site, the type and concentration of contaminant, as well as the treatment costs. Ex situ techniques are more expensive compared to in situ techniques because of the additional cost attributable to excavation. However, the cost of installing equipment on site and the inability to visualize and monitor the subsurface of contaminated sites are major concerns when conducting in situ bioremediation (Singh & Tripathi, 2023; Azubuike, Chikere & Okpokwasili, 2016). On the other hand, two technologies can also be applied to improve bioremediation: biostimulation (addition of nutrients) and bioaugmentation (addition of microorganisms), which are used when successful treatment is required and after previously performing a physical, chemical and microbiological characterization of the site to be bioremediated.

Mangrove forests are very complex ecosystems with multiple ecological functions and high economic value (Olguin *et al*., 2007). Given the ecological importance of the red mangrove as a protective barrier against natural phenomena, nesting and rearing of juvenile marine and coastal species (Lewis 2005), it will be necessary to determine whether the red mangrove (Rhizophora mangle) has the potential to be used as a phytoremediation plant for heavy metals, as well as to determine whether filamentous fungi such as T. harzianum, Rhizopus sp and the bacterium S. marcescens help in the phytoremediation of heavy metals (Lewis *et al*., 2005).

Con el desarrollo del presente trabajo se generará información importante para la fitorremediación de metales pesados presentes en suelos contaminados con petróleo crudo medio, el cual es representativo de la mezcla mexicana que se produce en la Sonda de Campeche, México. Por lo anterior, el objetivo de esta investigación fue fitorremediar metales pesados presentes en suelos contaminados con petróleo crudo medio (22.4 °API) utilizando plantas de mangle rojo (*Rhizophora mangle*) y un consorcio microbiano conformado por *Serratia marcescens, Trichoderma harzianum* y *Rhizopus* sp.

This paper contains the abstract, introduction, methodology, results, conclusions, acknowledgments and references. The introduction includes the objective and hypothesis of the work. The hypothesis of the work was the following: "With the application of Rhizophora mangle plants (phytoremediation) and the microbial consortium formed by Trichodema harzianum, Rhizopus sp and Serratia marcescens (bioaugmentation), it is expected to obtain high percentages of phytoremotion of heavy metals present in soils contaminated with medium crude oil". On the other hand, the methodology includes the evaluation of seedling height and root length of red mangrove seedlings; as well as the determination of physicochemical, microbiological and heavy metal parameters in the phytoremediation of soils contaminated with crude oil. The results include seedling height and root length of red mangrove seedlings, pH, electrical conductivity, texture and organic matter of the soil during the phytoremediation trial, in addition to the colony forming units of the microbial consortium and the percentage of phytoremediation of heavy metals in soils contaminated with crude oil.

2. Materiales y métodos

The experiment of phytoremediation of contaminated soil using red mangrove plants was installed in the botanical garden of the Universidad Autónoma del Carmen. The microorganisms that were used as a microbial consortium in the phytoremediation of heavy metals in oil contaminated soils were provided by Dr. Miguel A. Ramírez Elías, professor of the Faculty of Chemistry of the UNACAR. For the trial, a selection of Rhizophora mangle seedlings was made in order to have a homogeneous size. The experimental treatments were prepared by placing one kilogram of previously sterilized soil in crystallizers. To each soil unit, 30, 60 and 100 mg of crude oil were added, respectively, to obtain the respective units contaminated with 30, 60 and 100 ppm (mg/kg). Subsequently, they were placed in a sowing bag and transferred to a greenhouse of the botanical garden of UNACAR. Finally, the red mangrove seedlings previously selected for their size were placed in the sowing bag in triplicate and the prepared microbial consortium was applied to each of the experimental treatments. Likewise, the pH of the soil and sterilized water was adjusted to 6 to provide an optimum pH for the microbial consortium and the red mangrove seedlings used (Moreno, 2000).

2.1. Evaluation of the height and root length of red mangrove (Rhizophora mangle) seedlings

The following characteristics were evaluated in the seedlings: propagule height, root length and number of leaves. All this was done in each cutting or time period, respectively (0, 15, 30, 30, 60 and 90 days). A graduated ruler was used for each of the measurements. The objective was to know the changes of the seedlings with respect to the progress of the phytoremediation trial.

2.2. Determination of physicochemical, microbiological and heavy metal parameters during phytoremediation of crude oil contaminated soil

The soil samples obtained from each cut or time period were deposited in glass crystallizers for drying at 40 °C, in a Fisher Scientific oven. The dried soils were sieved with a mesh < 0.05 mm. From each sieved sample, the amount of soil necessary to determine the physicochemical parameters was taken; as well as the concentration of the following heavy metals: Cd, Cu, Ni, Pb, and Zn.

For the determination of physicochemical parameters such as pH and electrical conductivity (EC), the potentiometer and conductivity meter method was used, in accordance with the NOM-021-SEMARNAT 2000 standard. Organic matter was determined using the Walkey and Black (1999) method described in the official Mexican standard NOM-021-SEMARNAT-2000. Texture was determined using the Bouyoucos technique described in NOM-021-SEMARNAT-2000.

For the determination of microbial activity, 10 g of soil were taken from each experimental unit and added in an Erlenmeyer flask then 90 mL of distilled water was added, shaken until a suspension was formed and using the dilution and raking technique in Petri dishes with potato dextrose agar (PDA) and nutrient agar (AN), respectively; the colony forming unit (CFU) count was performed in each experimental unit of the phytoremediation trial (Aydin *et al*., 2017).

The determination of the concentration levels of heavy metals (Cd, Cu, Ni, Pb and Zn) was carried out using EPA (Environmental Protection Agency) method 3050B, which refers to the acid digestion of sediments, sludge and soils by Atomic Absorption Spectrophotometry (Lorentzen & Kingston, 1996). A Thermo Scientific Atomic Absorption Spectrophotometer, model iCE 3000 Series, was used. Standard solutions of Cd, Cu, Ni, Pb and Zn and INORGANIC VENTURES certified reference materials were used to determine the concentration of heavy metals. The calibration curve started with a 1000 mg/L solution for the five metals under study. All samples and the blank were prepared with deionized water. Finally, from the calibration curve for each metal, the samples were read and the concentration of heavy metals was determined (Handschuh, 2013).

For the determination of the percentage of heavy metal phytoremoval, the following equation was applied:

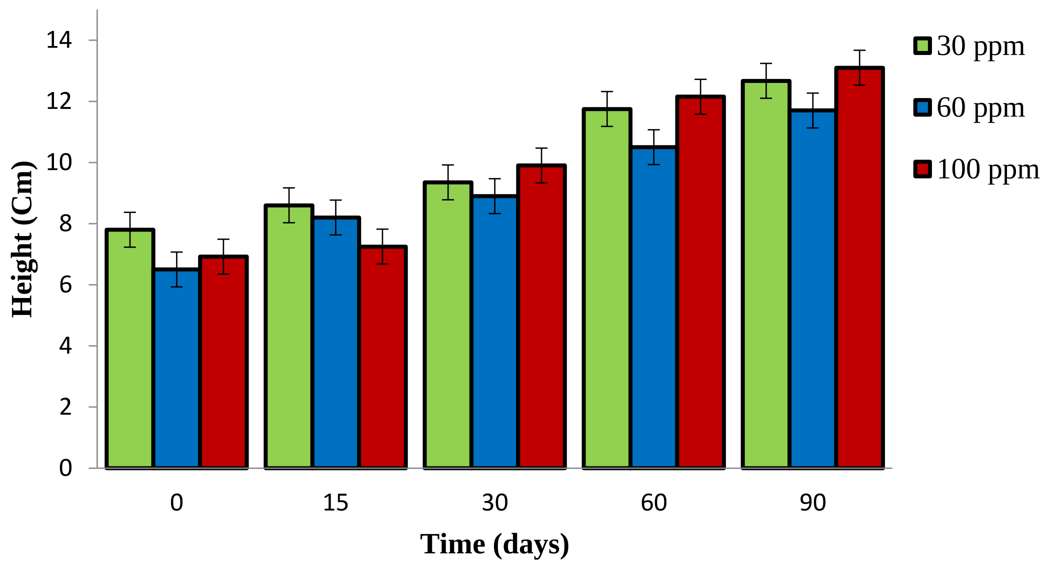
$$\%\, Fitorremediación = \frac{[metal\ en\ el\ testigo] - [metal\ en\ la\ muestra]}{[metal\ en\ el\ testigo]} * 100 \qquad (1)$$

3. Results

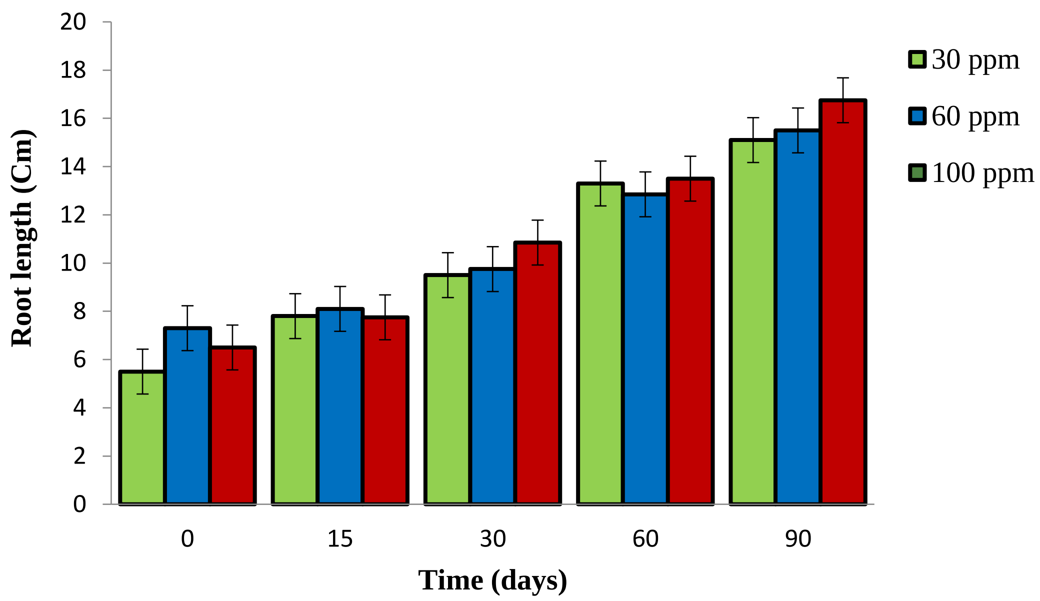
3.1. Seedling height and root length of Rhizophora mangle during phytoremediation.

In Figure 3.1, it is observed that the growth and height of the Rhizophora mangle seedlings was sequential and proportional in the three concentrations of oil and in the respective exposure times, presenting an average height of 9.69 cm, a minimum height of 6.50 cm in the 60 ppm concentration and a maximum height of 13.10 cm in the 100 ppm concentration in a period of 90 days. Likewise, the increase in root length in the monitored seedlings had a growth similar to the plant height, as shown in Figure 3.2. The average root length was 10.67 cm, starting with a minimum length of 5.50 cm at 30 ppm and reaching a maximum root length of 16.75 cm at 100 ppm. This confirms the rhizospheric strength of the red mangrove (Rhizophora mangle) and its application in phytoremediation processes of heavy metals present in soils or sediments contaminated with total petroleum hydrocarbons (Moreira *et al*., 2013).

Graph 3.1 Height of Rhizophora mangle seedlings), (Mean ± Standard Error)



Graph 3.2 Root length of Rhizophora mangle seedlings), (Mean ± Standard Error)

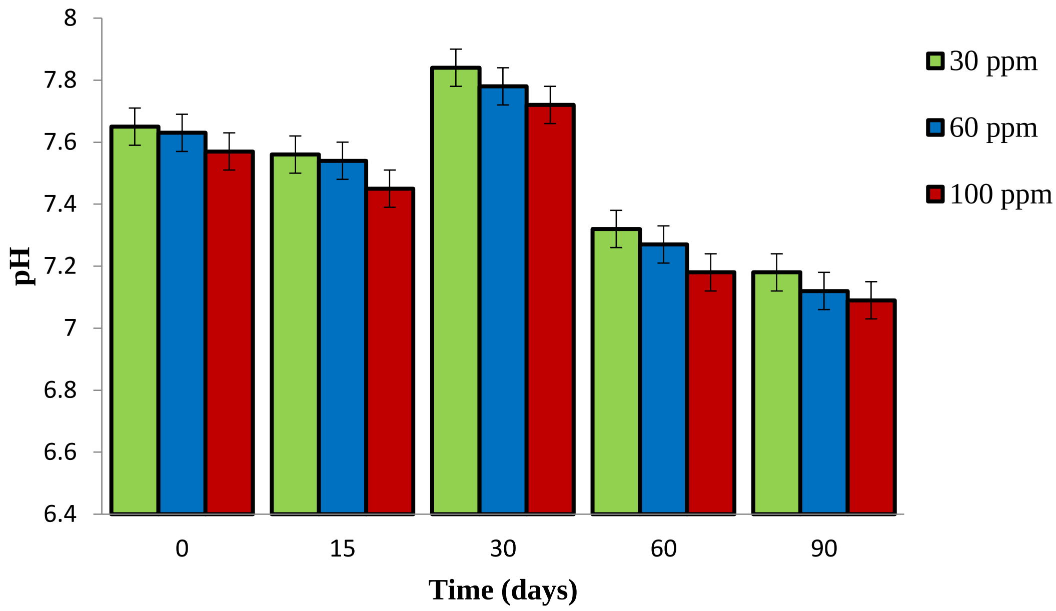


3.2. Soil pH during phytoremediation

The average pH value was 7.46; the minimum pH value was 7.09 at 90 days at the 100 ppm oil concentration and the maximum pH value was 7.84 at 30 days at the 30 ppm concentration (Graph 3.3.). This shows that the lower the concentration of oil in the soil, the higher the pH value, and at high oil concentrations the pH tends to neutralize. Changes in pH may be associated with the buffering capacity of the soil and active microbial metabolism in the presence of hydrocarbons and nutrients (Atlas and Bartha, 2001).

The average pH of 7.46 obtained in the present heavy metal phytoremediation study was lower compared to the average pH of 7.55 reported by Moreira *et al*. (2013) in a study evaluating the effects of metals on phytoremediation of total petroleum hydrocarbons for 90 days. Therefore, the obtained pH values are representative of mangrove soils and sediments, which favors the activity of microorganisms present in bioremediation and phytoremediation processes.

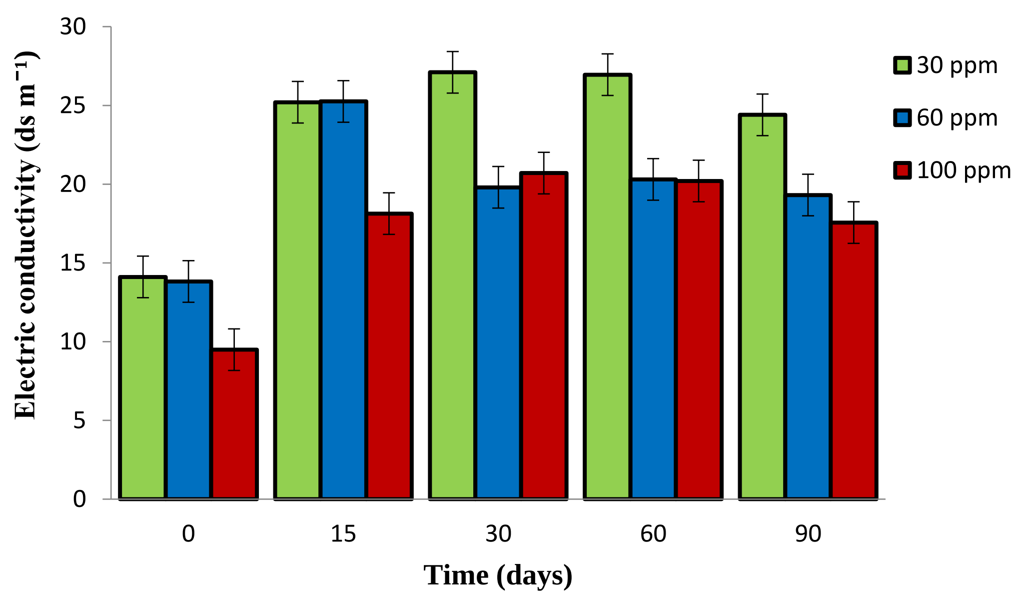
Graph 3.3 Soil pH (Mean ± Standard Error)



3.3. Soil electrical conductivity during phytoremediation

Graph 3.4 shows the data obtained from the experiment, where it can be observed that at the beginning of the test (0 days) there is the lowest value of electrical conductivity in the soil compared to the other cuts of the test (15, 30, 60 and 90 days); this can be attributed and associated with the initial concentration of oil, because as the phytoremediation test progressed the concentration of crude oil in the soil was reduced, which generated the increase of the EC in the soil after 15 days of the test.

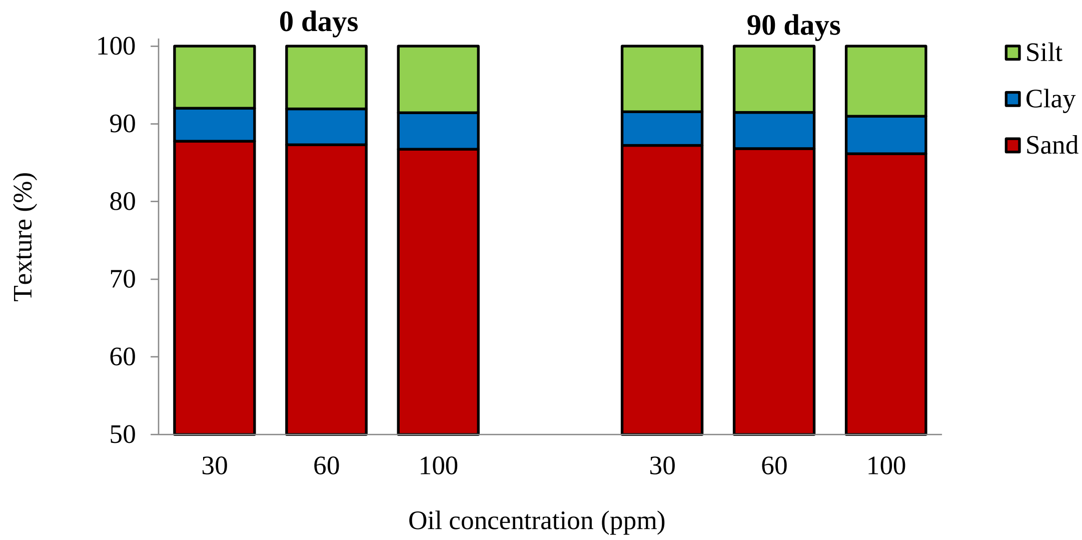
Graph 3.4 Electrical conductivity (EC) of the soil, (Mean ± Standard Error)



3.4. Soil texture during phytoremediation

Soil texture is another important factor that influences the mobility and availability of heavy metals (Castillo *et al.*, 2017). According to the results shown in Graph 3.5, it is observed that the soil texture is sandy at the beginning and end of the phytoremediation trial. The soil texture showed the following average values: sands (87 %), clays (4.57 %) and silts (8.43 %).

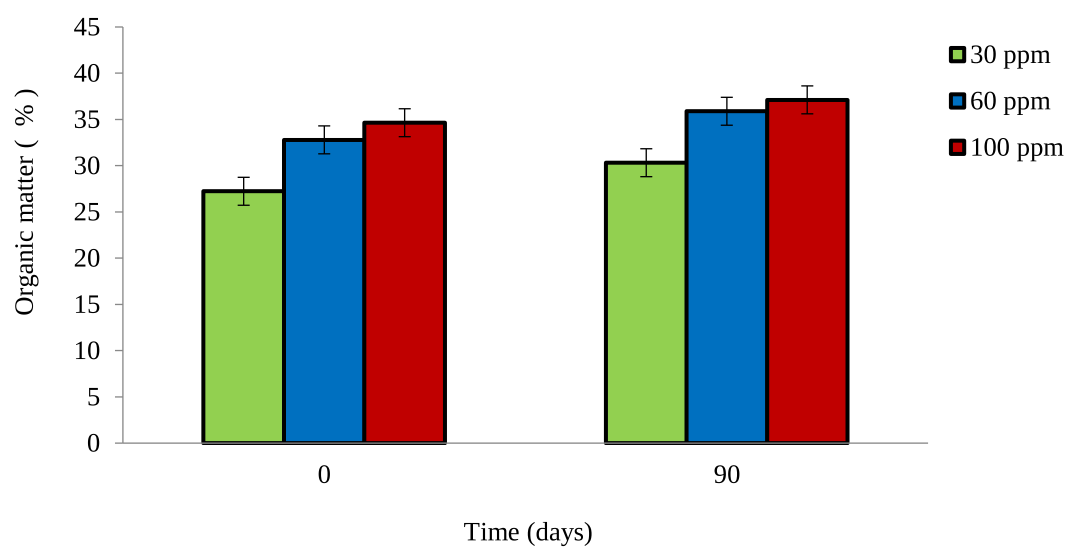
Graph 3.5 Texture of the soil used (%)



3.5. Soil organic matter during phytoremediation

Figure 3.6 shows the percentages of organic matter (OM) observed during the phytoremediation trial. When comparing the initial cut (0 days) with the final cut (90 days), an increase in organic matter can be observed for each concentration. The average organic matter value was 33%. This percentage is a very high value of OM. However, mangrove soils are very rich in organic matter and this is attributed to the fact that OM acts as a storehouse of metallic elements; in addition to its attraction for heavy metals that are in solution (Pineda, 2004).

Figure 3.6 Percentage of organic matter in soil, (Mean ± Standard Error)



3.6. Microbiological parameters of the microbial consortium during phytoremediation

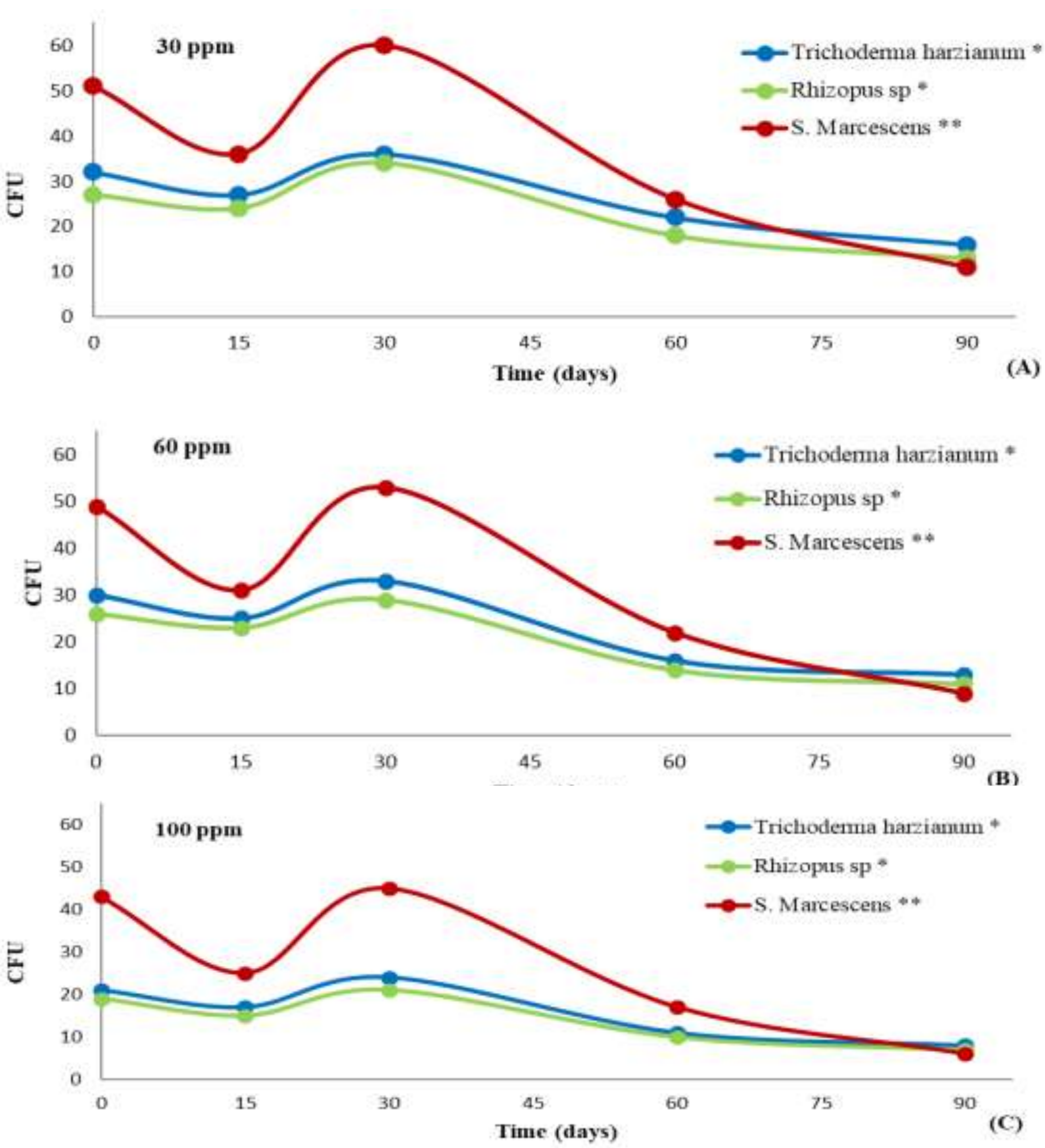
In order to know the content of colony forming units (CFU), the dilution and raking technique was applied in plates in each period of time and concentration of oil present in the soil. The results of the CFU count obtained from the microbial consortium formed by Trichoderma harzianum, Rhizopus sp and Serratia marcescens are shown below.

Figure 3.7 shows a comparison of the colony forming units of the microbial consortium, as well as the behavior and number of CFU for each concentration (30, 60 and 100 ppm) used in the phytoremediation.

It should be noted that the colony forming units of the fungi are expressed as CFU x 10^3 and for the bacteria as CFU x 10^6; taking this into account, it can be observed that the bacteria (Serratia marcescens) show the highest number of CFU compared to the filamentous fungi (Trichoderma harzianum, Rhizopus sp). Kotoky & Pandey (2020) indicated that the presence of the bacterium Serratia marcescens favors the activity of the enzyme glutathione-S-transferase in mangrove plants, which favors plant growth and reduces stress in the presence of heavy metals. This confirms their behavior in the present study, since the red mangrove plants apparently did not inhibit their height and root length during the heavy metal phytoremediation trial. The CFU obtained in the present study at 30 ppm with Serratia marcescens at 30, 60 and 90 days (60 x 106 CFU g-1, 27 x 106 CFU g-1 and 10 x 106 CFU g-1) were slightly higher than those reported by Moreira *et al*. (2013) in a study evaluating the effects of metals in the phytoremediation of 33 ppm total petroleum hydrocarbons at 30, 60 and 90 days (24.4 x 106 CFU g-1, 32 x 106 CFU g-1 and 7 x 106 CFU g-1).

The microbial consortium had the same behavior and growth trend including the general increase observed at 30 days of the trial, due to the second bioaugmentation of the consortium, with the purpose of guaranteeing the CFU of bacteria and fungi until the end of the trial, given that with the passage of time the consortium tends to decrease its growth due to the reduction of nutrients in the soil.

Gráfico 3.7 Unidades Formadoras de Colonias (UFC) del consorcio microbiano: **A**) 300 ppm, **B**) 60 ppm y **C**) 100 ppm, Las UFC para Hongos (*) se expresan como UFC x 10^3 y para la Bacteria (**) se expresan como UFC x 10^6.



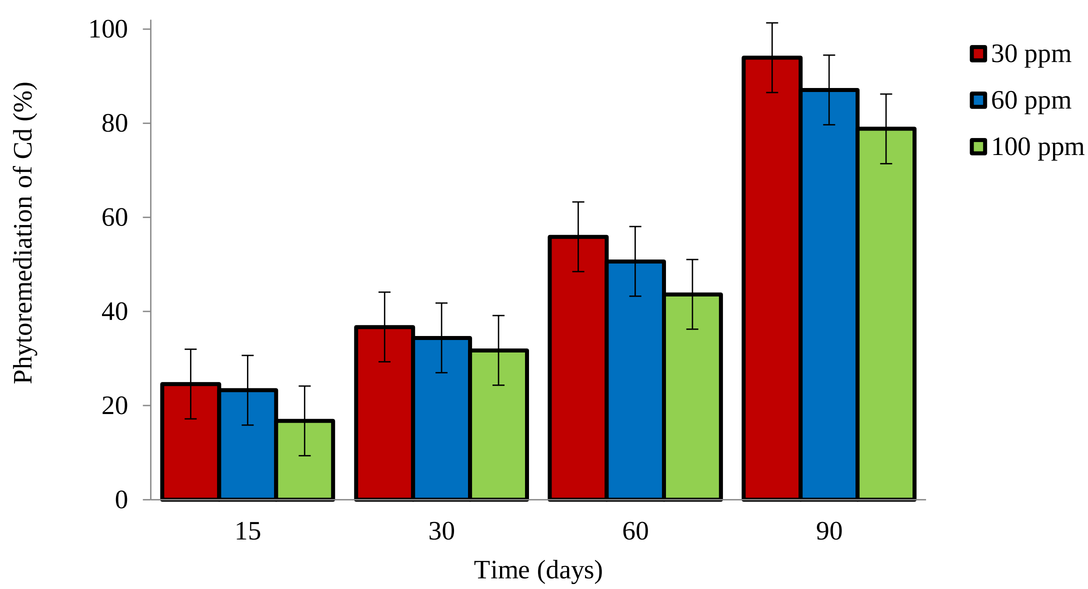
3.7 Percentage of phytoremoval of heavy metals (Cd, Cu, Ni, Pb and Zn) in soils contaminated with crude oil

3.7.1 Cadmio (Cd)

Figure 3.8 shows each of the percentages of cadmium phytoremoval in the respective time periods and concentrations. The percentages varied depending on the concentration; it was observed that the lower the oil concentration, the higher the percentage of cadmium phytoremoval. At the end of the trial (90 days) it can be observed that, at 30 ppm of crude oil, an average of 93 % Cd phytoremoval was obtained, as opposed to the concentration of 60 ppm, where 87 % Cd phytoremoval was obtained and for 100 ppm of oil the Cd phytoremoval was 78 %. Finally, in the present trial the average Cadmium phytoremoval was 86 %.

The 93 % phytoremediation of Cd obtained in the present study at 30 ppm crude oil at 90 days of the trial was higher compared to the 35 % phytoremediation of Cd reported by Moreira *et al*. (2013) in a study evaluating the effects of metals on the phytoremediation of 33 ppm total petroleum hydrocarbons for 90 days. Therefore, the phytoremediation of Cd in this study was approximately 58 % more efficient in the phytoremediation of Cd.

Graph 3.8 Percentage of phytoremediation of Cadmium (Cd), (Mean ± Standard Error), (Mean ± Standard Error)

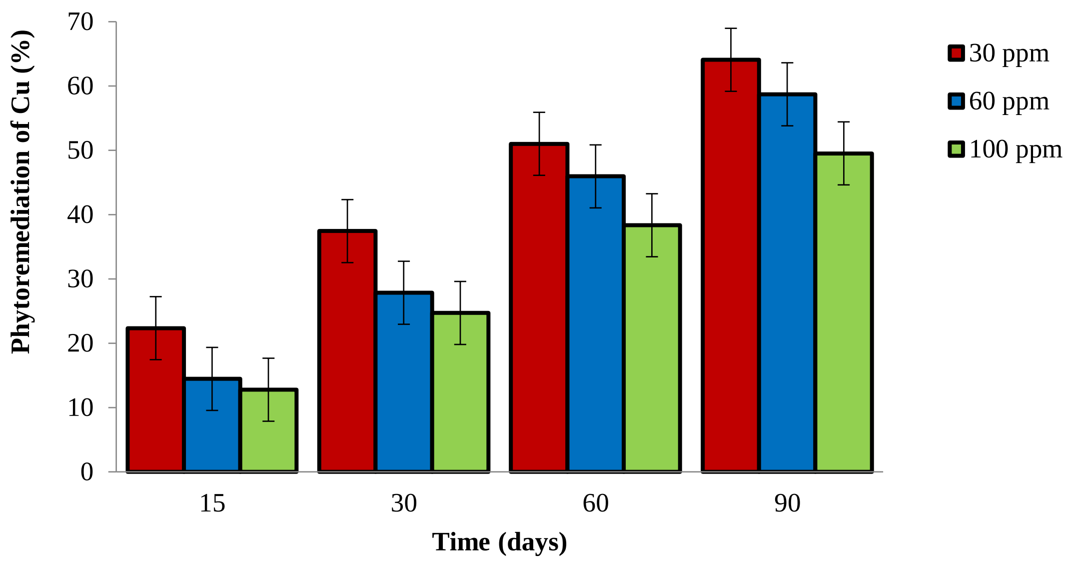


3.7.2 Copper (Cu)

The phytoremoval percentages for copper can be seen in Graph 3.9, in which a constant Cu phytoremoval is observed in each cut or period of time. It can be observed that for 100 ppm of oil, 49 % of Cu phytoremoval was obtained and for the 60 ppm concentration, 59 % of Cu phytoremoval was obtained. Finally, for the 30 ppm concentration, it presented 64% and was the highest phytoremoval value at 90 days. Finally, in the present trial, the average phytoremediation of copper was 57.3 %.

The 64% Cu phytoremediation obtained in the present study at 30 ppm crude oil at 90 days of the trial was higher compared to the 25% Cu phytoremediation reported by Moreira *et al*. (2013) in a study evaluating the effects of metals on the phytoremediation of 33 ppm total petroleum hydrocarbons for 90 days. Therefore, the Cu phytoremediation in this study was approximately 39 % more efficient in the phytoremediation of Cu.

Graph 3.9 Percentage of Phytoremediation of Copper (Cu), (Mean ± Standard Error))

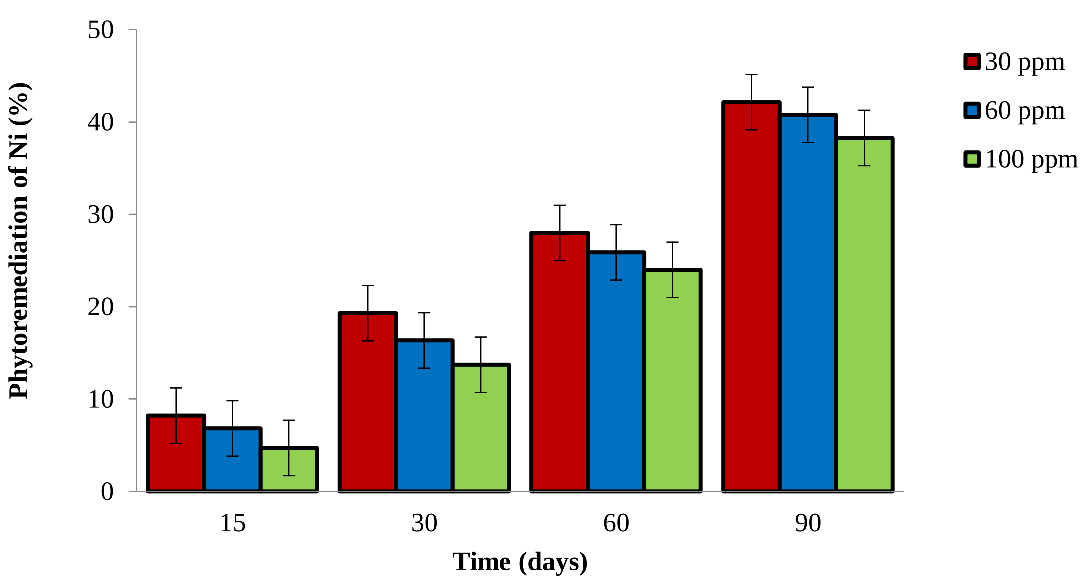


3.7.3 Nickel (Ni)

Figure 3.10 shows the phytoremoval values of Nickel obtained in the experiment. It can be observed that as the exposure time passes, the phytoremotion percentage increases, this is due to the adaptation of the Rhizophora mangle seedlings and the function of the microorganisms showing a constant phytoremotion. The achieved percentages of Ni were as follows: 42 % phytoremotion at 30 ppm, 40 % at 60 ppm and 38% for 100 ppm in the last cut, presenting a variation of 2 % between concentrations. Finally, in the present trial the average phytoremediation rate for Nickel was 40 %.

The 42% phytoremediation of Ni obtained in the present study at 30 ppm crude oil at 90 days of the trial was lower compared to the 57% phytoremediation of Cu reported by Moreira *et al*. (2013) in a study evaluating the effects of metals on the phytoremediation of 33 ppm total petroleum hydrocarbons for 90 days. Therefore, the phytoremediation of Ni in this study was approximately 15 % less efficient in the phytoremediation of Ni.

Graph 3.10 Percentage of Phytoremediation of Nickel (Ni), (Mean ± Standard Error)

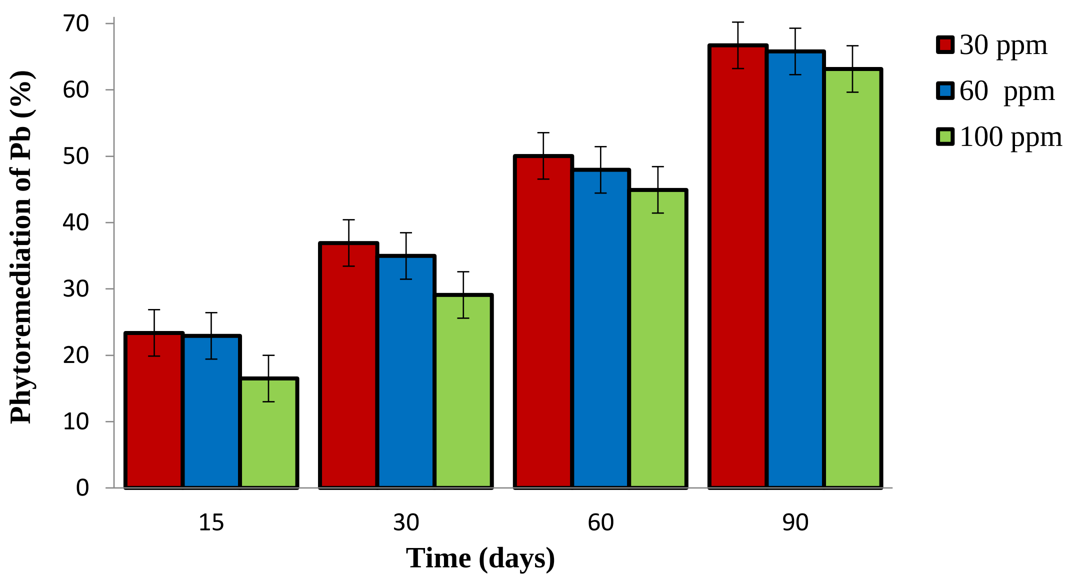


3.7.4 Plomo (Pb)

The phytoremoval percentages for lead were calculated, which are shown in Graph 3.11, obtaining as a result that the three concentrations do not show much difference in the phytoremoval percentages of Pb. The percentages obtained are as follows: for 30 ppm, 66.5 % phytoremoval was obtained, at 60 ppm 65 % and for 100 ppm of oil 63 % of Pb phytoremoval. Finally, in the present trial the average phytoremoval of Lead was 64.8 %.

The 66.5 % phytoremediation of Pb obtained in the present study at 30 ppm crude oil at 90 days of the trial was slightly lower compared to the 66.9 % phytoremediation of Cu reported by Moreira *et al*. (2013) in a study evaluating the effects of metals on the phytoremediation of 33 ppm total petroleum hydrocarbons for 90 days. Therefore, the phytoremediation of Pb in this study was approximately 0.4 % less efficient in the phytoremediation of Pb.

Graph 3.11 Percentage of Phytoremediation of Lead (Pb), (Mean ± Standard Error)

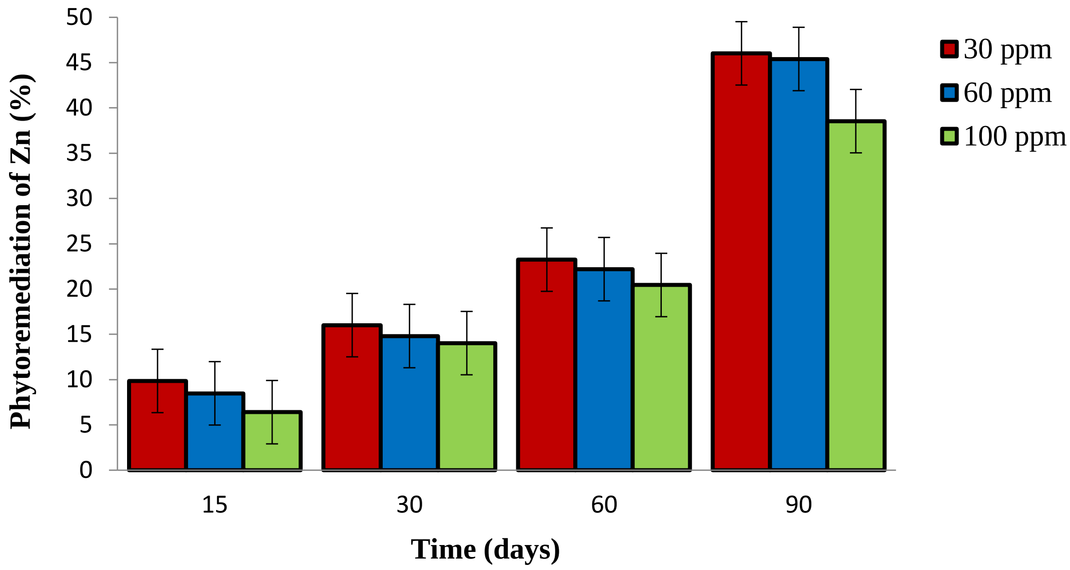


3.7.5 Zinc (Zn)

Finally, Graph 3.12 shows the phytoremediation percentages of Zinc obtained for each cut or time period. The phytoremediation percentages of Zn, as with the metals indicated above, tend to increase with the passage of time, as well as varying according to the concentrations used in the experiment. The 30 ppm and 60 ppm concentrations show a small variation in their percentages, obtaining 46 % and 45 % respectively, in contrast to the 100 ppm crude oil concentration where 38 % of Zn phytoremoval was obtained in a period of 90 days. Finally, in the present trial, the average Zinc phytoremediation rate was 43 %.

The 46 % phytoremediation of Zn obtained in the present study at 30 ppm crude oil at 90 days of the trial was higher compared to the 43 % phytoremediation of Cu reported by Moreira *et al*. (2013) in a study evaluating the effects of metals on the phytoremediation of 33 ppm total petroleum hydrocarbons for 90 days. Therefore, the phytoremediation of Zn in this study was approximately 3 % more efficient in the phytoremediation of Zn.

Graph 3.12 Percentage of Zinc (Zn) Phytoremediation, (Mean ± Standard Error), (%)



After 90 days, the present phytoremediation trial presented the following order and average phytoremediation percentages of heavy metals: Cd (86 %) > Pb (65 %) > Cu (57 %) > Zn (43 %) > Ni (40 %). Therefore, cadmium was the metal that obtained the highest average phytoremediation percentage of heavy metals present in soils contaminated with medium crude oil, which is in agreement with Pittarello *et al*., (2018) highlighting that cadmium is often found in high concentrations in sediment or mangrove soils.

Finally, the average phytoremoval percentages of heavy metals achieved with red mangrove (Rhizophora mangle) in the present work, presented the following order: Cd > Pb > Cu > Zn > Ni; which, suggests the potential of Rhizophora mangle as a phytoremediation plant for such heavy metals together with the microbial consortium formed by Trichoderma harzianum, Rhizopus sp and Serratia marcescens.

4. Conclusions

High percentages of heavy metal phytoremoval were obtained in soil contaminated with medium crude oil, using Rhizophora mangle seedlings and a microbial consortium composed of Trichoderma harzianum, Rhizopus sp and Serratia marcescens. Therefore, the hypothesis is accepted.

It is also concluded that the presence of the microbial consortium favorably influenced the growth of the red mangrove plants and their function as a phytoremediation plant. Therefore, the red mangrove (Rhizophora mangle) has the potential to be used as a phytoremediation plant for heavy metals together with the microbial consortium formed by T. harzianum, Rhizopus sp and S. marcescens.

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