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BIOREMEDIATION OF CRUDE OIL-BEARING SOIL: EVALUATING THE EFFECT OF RHAMNOLIPID ADDITION TO SOIL TOXICITY AND TO CRUDE OIL BIODEGRADATION EFFICIENCY

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ABSTRACT
This work is aimed at evaluating the potentiality of adding a rhamnolipid biosurfactant in a petroleum-bearing soil. For this purpose, dehydrogenase activity and seed germination (*Lactuca sativa*) testes were performed before the biodegradation assays with different concentrations of rhamnolipid (1 to 15mg for 1g of soil). The addition of 1 and 15 mg g^{-1} of rhamnolipid was harmful to the soil environment. The biodegradation assays were carried out at room temperature during 45 days in bioreactors containing 450g of a polluted soil with different rhamnolipid concentrations varying from 1 to 15 mg g^{-1}. The nutrients were corrected through the addition of NH_4NO_3 and KH_2PO_4, in a nutritional ratio of C:N:P=100:15:1. The humidity was adjusted to 50% of the liquid retention capacity. Besides these assays, a control test was conducted without adding rhamnolipid. TPH (Total petroleum hydrocarbon) removal and seed germination were evaluated at the end of these experiments. When 4 mg g^{-1} of rhamnolipid were used a TPH removal of about 60% was observed. The biosurfactant addition improved all treatments, except for the assays with addition of 1 and 15 mg g^{-1} in which a decrease of the bioremediations rates was observed in the toxicity tests.

KEYWORDS: Bioreactors, biodegradation, biosurfactant, petroleum, *Lactuca sativa*, dehydrogenase activity.

1. INTRODUCTION

Pollution caused by petroleum and its derivatives is the most prevalent problem in the environment. The release of crude oil into the environment by oil spills is receiving worldwide attention. Many accidents can cause soil pollution and, for this reason, many techniques are being developed to clean up petroleum contaminated soil. The biological treatments are more efficient and cheaper than chemical and physical ones. In relation to biological treatment, the bioremediation technology is being employed for the degradation of crude oil in soil matrix through microorganisms able to transform petroleum hydrocarbons in less toxic compounds. However, the low solubility and adsorption are two major properties of high molecular weight hydrocarbons that limit their availability to microorganisms. In this case, the addition of biosurfactant enhances the solubility and removal of these contaminants, improving oil biodegradations rates.

Biosurfactants are metabolic products of bacteria and fungi and they are classified in agreement with their chemical composition and/or microbial origin. These biomolecules are produced mainly by aerobic growth of microorganisms in aqueous phase containing carbohydrates, hydrocarbons, or a mixture of them as carbon source. In general, the biosurfactants are neutral or anionic, varying from small fat acids to large polymeric

structures. They are aliphatic molecules that possess a polar group (hydrophilic) and a non-polar group (hydrophobic) and, according to the structure, they tend to interact amongst themselves forming clusters known as micelle. Usually, the hydrophobic group is composed of hydrocarbons with 10 to 18 atoms of carbon, and the hydrophilic part is quite diverse, being a carbohydrate, ester, hydroxyl, phosphate, or a carboxylic group (Bognolo, 1999). Biosurfactants possess better properties than many chemical surfactants, bearing in mind that they usually possess low CMC (Critical Micelle Concentration) values and interfacial tension in aqueous solution (Banat, 1995). Besides their low CMC values, they stand out by having other properties as tolerance to temperature and pH changes, high hydrocarbon emulsification, solubilisation indexes, low toxicity, and high biodegradability (West and Harwell, 1992; Nitschke and Pastore, 2002). However, the interactions between biosurfactants and crude oil, surfactants and soil components and between biosurfactants and the soil microorganisms should be well investigated. The investigation of the biosurfactants toxicity in soils is extremely important to foresee the impact of their addition to the environment. The ecotoxicity bioassays are analytical methodologies that allow toxicity characterization of chemical substances. The exposition of bioindicators to those substances is a valuable tool for environmental analysis. For toxicity tests in soil, the interactions between the chemical compounds and the soil should be taken into account to correctly predict the chemical impact in the atmosphere. Soil is a key-component of the environment and, depending on the type of mineral, organic matter, pH, redox potential, humidity and soil handling, the pollutants can be adsorbed or released, causing different poisonous effects (Kapanen and Itavaara, 2001). For terrestrial ecotoxicity bioassays some methodologies have been used and, in this work, the seed germination with *Lactuca sativa* and enzymatic tests with dehydrogenase were performed.

The enzymatic activity can be used to describe the effects of poisonous compounds on the soil microbiological population. The enzymes used in the soil microbiological activity are the hydrolases (phosphatases and ureases) and the oxidoreductases (dehydrogenases) (Ratsep, 1991). ATP measurements are used for biomass quantification as well as microbial activity. As microbial cells lose ATP when they die, the measurement of ATP concentration gives an estimation of live biomass. The determination of the dehydrogenase activity is the most common method used for enzymatic toxicity tests. This method is based on TTC (hydrochloric triphenileltrazolium) and TPF (triphenil formazan) reduction rate in the soil after incubation at 30ºC for 24 h. TTC is used as final electron acceptor, being therefore, one of the most frequently used methods for such estimation (Bitton and Koopman, 1992).

There are several types of toxicity studies involving plant processes. According to Fletcher (1991), the tests with plants can be used in 5 different categories: biotransformation, food chain uptake, sentinel, surrogate, and phytotoxicity. Among those tests, the phytotoxicity is receiving more attention during the last years. Some species recommended by the OECD (1984); USEPA, and FDA (Fletcher, 1991) are radish (*Raphanus sativus*); carrot (*Daucus carota*); rice (*Oryza sativa*); turnip (*Brassica rapa*); soybean (*Glycine max*); oats (*Avena sativa*); cabbage (*Brassica campestri*); corn (*Zea mays*); tomato (*Lycopersicon esculentum*); bean (*Phaseolus aureu; Phaseulus vulgari*); onion (*Allium cepa*); sorghum (*Sorghum bicolor*), and lettuce (*Lactuca sativa*). Germination studies are considered short-term and primarily assess acute toxicity effects. In germination studies, seeds are planted in a small quantity of the contaminated soil, and seedlings counted after an extended incubation period. Results are compared to seedling enumeration in uncontaminated control soil. Seed germination has been shown to decrease significantly in heavy metal and hazardous contaminated soil. Plants that are sensitive to poisonous substances can be used as bioindicators (Banks and Schultz, 2005).

As mentioned above, the behavior of the biosurfactant as auxiliary of bioremediation and toxicity of petroleum contaminated soil should be investigated. For this reason, the aim of this work was to investigate the use of biosurfactant as auxiliary of bioremediation technology as well as to investigate the efficiency and toxicity of a rhamnolipid in a petroleum contaminated soil.

2. EXPERIMENTAL

2.1 Soil, Crude oil, and biosurfactant

In this work, soil of the Northeast area of Brazil was used. This soil was homogenized, sieved, and further contaminated with 50 mg g^{-1} of crude oil. The chemical, physical, and biological characteristics of the soil are shown in Table 1.

The crude oil was classified as paraffinic oil, with the following characteristics: sulfur (S) = 0.44%; carbon (C) = 86.2%; hydrogen (H) = 12.3%; nitrogen (N) < 0.3% (below the detection limit). A rhamnolipid biosurfactant was employed since this compound is being widely used to enhance bioremediation processes (Noordman *et al.,* 2002; Nitschke and Pastore, 2002; Mulligan*,* 2005).

Table 1. Soil characteristics

Parameter	Value	Parameter	Value
N (g kg^{-1})	0.3	Particle Dens. (g ml^{-1})	1.5
P (g kg^{-1})	0.075	Apparent Density	1.3
$C_{organic}$ (%)	5.3	Porosity (%)	20
Organic Matter (%)	9.2	LRC [(1)] (%)	28
pH	6.4	THB[(3)] (CFU g^{-1})	2.3 x 10^6
TPH[(2)] (mg g^{-1})	46	BH[(4)] (cell g^{-1})	2.1 x 10^3

[(1)]LCR = Liquid retention capacity; [(2)]Total petroleum hydrocarbon [(3)]THB = Total heterotrophic bacteria- CFU = Colony Forming Unit; [(4)]HB = Hydrocarbonoclastic bacteria;

2.2 Ecotoxicity test

Dehydrogenase activity: This technique was performed according to the Alef and Nanipiere (1995) method, using methanol as extractor. This test was accomplished in five replicates. Five milliliters of TTC (hydrochloric tripheniletrazolium) added, remained incubated for 24 h in an oven at 30ºC. TPF (tri-phenyl formazan) was extracted in 40mL of methanol, and after filtration the liquid phase was read in a spectrophotometer at 485 nm.

Germination Test: The method of seed germination and growth, suggested by Reis (2003), was employed using lettuce seeds of the *Lactuca sativa* species. The extract of the soil samples was placed in Petri dishes. Ten seeds of *L. sativa* were distributed in the dishes, equally spaced. The plates were incubated at 24ºC for 120 hours. After this time, the number of germinated seeds was counted and the elongation of the roots was measured from the transition point among the hypocolite to its extremity. The germination index (%GI) could be calculated through the following equations:

$$\%IG = \frac{(\%SG)x(\%GR)}{100} \quad (1)$$

$$\%SG = \left(\frac{\%EG}{\%CG}\right)x100 \quad (2)$$

$$\%GR = \left(\frac{GERm}{GERCm}\right)x100 \quad (3)$$

Where: % SG = seed germination; % GR = Growth of the root; % EG = Germination of contaminant; %CG = Control germination; GERm = elongation of root of the contaminant (Media); GERCm = Elongation of control root (media).

2.3 Biodegradations test

The experiments were performed in 20 cm high and 5 cm-diameter bioreactors using 3cm of support rock layer. The tests were maintained at room temperature using 3 l h^{-1} of a humid airflow. Figure 1 shows an outline of the reaction system for the biodegradation tests. The tests were accomplished by adding different concentrations of biosurfactant to different assays. The concentration of the rhamnolipid varied from 1 to 15 mg g^{-1}. The nutrients concentrations were corrected through the addition of ammonium nitrate (NH_4NO_3) and bi-hydrogen potassium phosphate (KH_2PO_4) using a nutritional ratio of C:N:P = 100:15:1. The tests were carried out for 45 days. During the experimental period the humidity was controlled through thermo-gravimetric method.

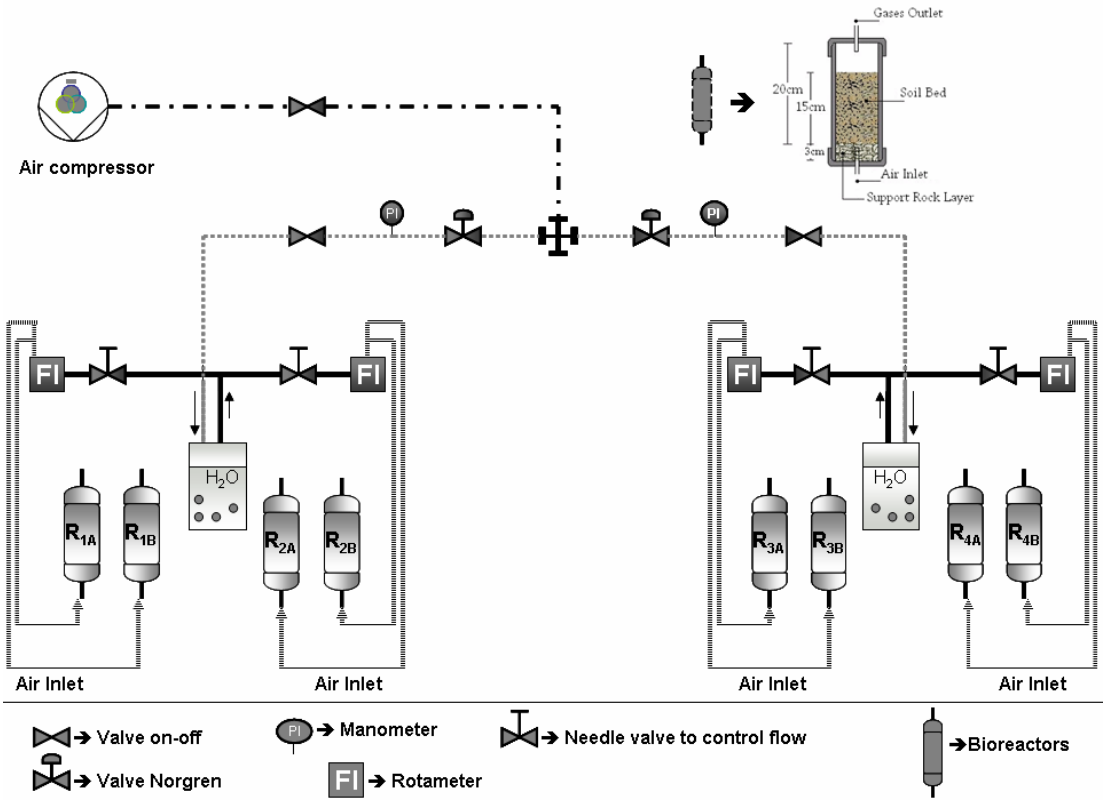

Figure 1. Outline of the experiments with aerobic fixed bed bioreactor

2.4 Analytical methodology

Total Petroleum Hydrocarbon (TPH): The methodology consists of extracting the hydrocarbons from the soil. A measured weight of dry soil, 0.2 g, was mixed to 10 ml of a specific solvent (S-316 for HORIBA). The extraction was accomplished in an ultrasound during 2 h. After extraction, the solution was filtered with 2 g of silica gel (60 to 200 meshes) and this filtered solution was analyzed by an infrared spectrometer (Horiba OCMA-350).

Quantification of microbial population: The quantification of the total heterotrophic microbial population was performed through the Pour Plate technique, using TSA (Trypic Soy Agar). A 5 g of soil sample was mixed to 50 ml of saline solution and kept in shaker for 1 hour at 30 ºC. The results were expressed as CFU g^{-1} of soil. The quantification of the hydrocarbonoclastic bacteria population was done by the most probable number technique (MPN) (Oblinger and Koburger, 1975), using the liquid mineral medium described by Vecchioli *et al.* (1990). The methodology employed was in agreement to Volpon *et al.* (1998).

3. RESULTS AND DISCUSSION

3.1 Evaluation of the surfactant concentrations in the soil before the biodegradation test

The wider use of enzymatic tests in toxicity testing requires standardization (Nannipieri *et al*., 1990). Studying inducible enzyme metabolism can be considered a sensitive indicator for detecting the effects of harmful compounds. In ecological studies, correlations have been determined between dehydrogenase activity, chemical composition, and structure of soil (clay, humus, nutrients, soil type and structure, and pH) or the concentrations of harmful compounds (Kapanen and Itakvaara 2001). Figure 2A shows the results of the dehydrogenase activity in the soil with and without crude oil after the addition of a rhamnolipid. Regarding the soil without crude oil, the activity increased until the concentration of 4 mg g^{-1} of rhamnolipid (~75 µg g^{-1} of TPF). However, after that concentration the microbial activity decreased. Nevertheless, for all concentration of rhamnolipid (1 to 15 mg g^{-1}) the activity did not reach results below the natural soil (without rhamnolipid - 6.5 µg g^{-1} of TPF), characterizing a tolerance of the microorganisms activity. An accentuated decrease of the dehydrogenase activity by the addition of rhamnolipid in a crude oil contaminated soil was observed, showing that with crude oil, the rhamnolipid can be extremely toxic to soil enzymatic activity, since the addition of the biosurfactant can enhance the bioavailability of crude oil (Mueller *et al.,* 1989). The high bioavailability could have affected soil microorganisms. However, the crude oil was already detrimental to soil activity, since there was a drop from 6.5 to 4.6 µg g^{-1}.

Plants are essential primary producers in the terrestrial ecosystem. Therefore, it is important to identify potential phytotoxins and understand the magnitude of their impact on different terrestrial ecosystems, using different pollutants. Lettuce is an important agricultural crop and it is fairly sensitive to toxic chemicals, which led to the widespread use of *Lactuca sativa* for toxicity tests (Oleszczuk, 2008). The increase of the rhamnolipid concentration decreased the germination index (GI) and the acute toxicity (EC50 values), *L. sativa* was between 4 and 6 mg g^{-1} (Figure 2B). Nevertheless, at the lowest concentration (1 mg g^{-1}) an increase of the GI was observed, reaching a value above the natural soil, which suggests that low concentrations may increase the availability of some nutrients improving soil fertility. The concentrations of 10 and 15 mg g^{-1} have shown a negative effect with about 80% of inhibition in comparison to the control (natural soil). This fact indicates that those concentrations are very damaging for the *L. sativa*. The addition of rhamnolipid in a crude oil contaminated soil was very toxic to the seed germination as observed by Banks and Schults (2005) since lettuce is very sensitivity to petroleum contaminants.

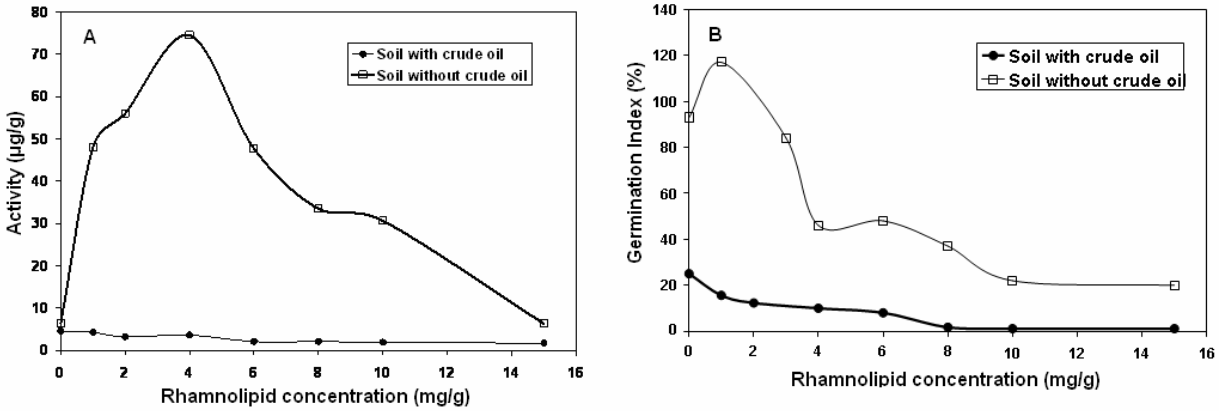

Figure 2. Dehydrogenase activity (A); germination index (B) in the different biosurfactant concentrations addition

3.2 Evaluation of crude oil biodegradation in soil in different biosurfactant concentrations

In spite of being toxic to crude oil contaminated soil as observed in Figure 2, the addition of rhamnolipid was investigated for biodegradation assays; since there is a consensus that using biosurfactant in petroleum contaminated soil can enhance the biodegradation assays (Deschênes *et al.,* 1996; Rojas-Avalizapa *et al.*, 2000; Cubitto *et al.*, 2005; Millioli *et al.*, 2005). Figure 3 shows the results of TPH removal (A), dehydrogenase activity (B), and germination index (C) during 45 days of biodegradation assays. Different rhamnolipid concentrations were added, comparing to the control test (without biosurfactant).

The addition of 4 mg.g^{-1} of rhamnolipid showed higher TPH removal (60%), despite being toxic at the beginning of the treatment (Figure 3A), however, a better activity (Figure 3B) and high seed germination (Figure 3C) was observed after 45 days. The treatments with addition of 10 and 15 mg.g^{-1} showed better results in relation to the control tests. At the beginning of the treatment the addition of rhamnolipid has a negative impact to the soil activity and seed germination, although, along the treatment process, soil microorganisms become more adapted which improves the biodegradation of crude oil. In fact, surfactants can enhance the mobility, solubility and bioavailability of hydrocarbon for microbial remediation (Zhou and Zhu, 2008).

Table 2 shows the microbial population before and after biodegradation assays. Conditions 4, 6, and 8 mg g^{-1} showed higher total heterotrophic bacteria (THB) and hydrocarbonoclastics bacteria (HB) after 45 days of experiment in bioreactors. The 1, 2, and 10 mg g^{-1} concentrations did not increase the microbial population, although the concentration of 15 mg g^{-1} was the only one that decreased the microbial population in comparison to the initial, suggesting a non tolerance of the microbial population to that concentration.

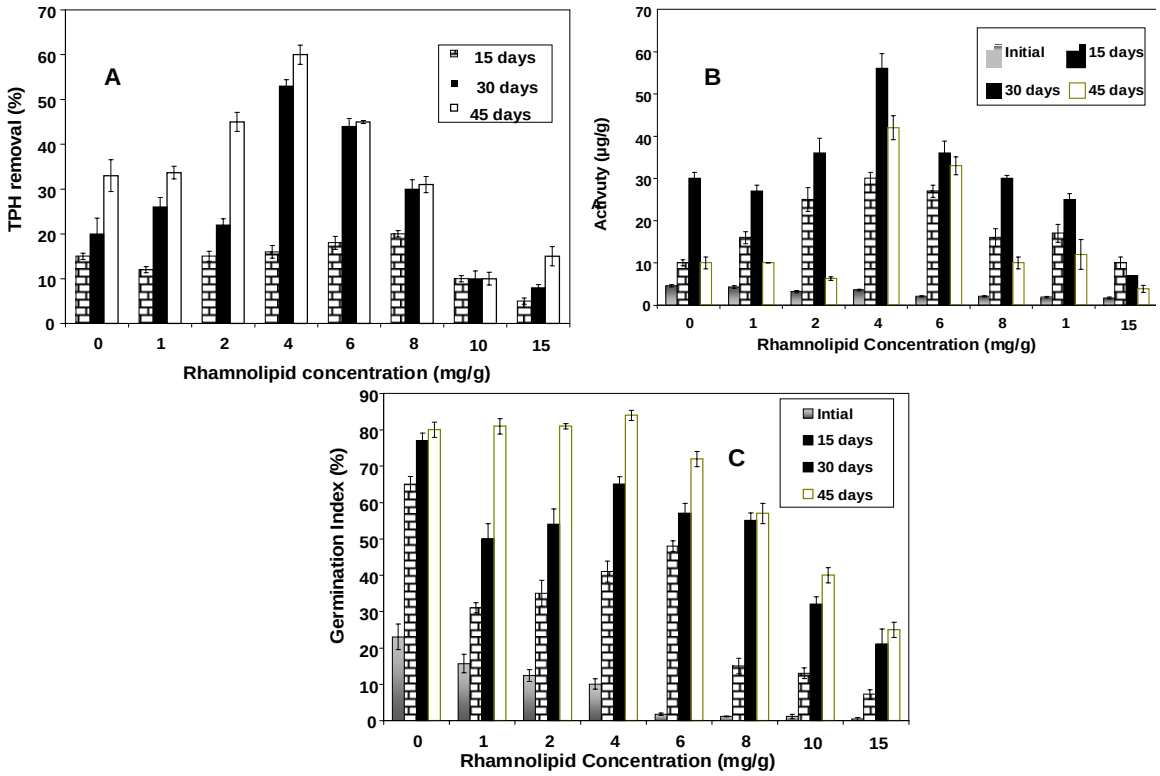

Figure 3. TPH removal (A), dehydrogenase activity (B), seed germination (C) in different rhamnolipid concentrations during the biodegradation assays

Table 2. Microbial population in different conditions before and after 45 days of experiments

Experiments	MICROBIAL POPULATION			
	Total heterotrophic bacteria (THB)		hydrocarbonoclastics bacteria	
	TIME		TIME	
Rhamnolipid (mg g^{-1})	*Initial*	*45 days*	*Initial*	*45 days*
0	7.4×10^6	5.3×10^6	6.2×10^3	1.7×10^3
1	2.8×10^6	2.3×10^6	3.3×10^3	1.9×10^3
2	1×10^6	5×10^8	2.3×10^3	6.8×10^5
4	8.2×10^6	6.2×10^{10}	2.3×10^3	6.8×10^5
6	7.8×10^6	1.3×10^{10}	8.6×10^3	3.3×10^5
8	7.3×10^6	3.1×10^7	2.4×10^3	8.2×10^4
10	5.1×10^6	5×10^6	6.2×10^3	2.7×10^3
15	3.1×10^6	3×10^5	3.2×10^3	8.5×10^2

4. CONCLUSIONS

The rhamnolipid toxicity in a soil without crude oil indicated that there is an optimum rhamnolipid concentration, which was 4 mg g^{-1} of rhamnolipid for dehydrogenase activity. As the concentration of rhamnolipid increased, the seed germination decreased with *Lactuca sativa (*lettuce). However, the addition of rhamnolipid in a crude oil contaminated soil suggested high toxicity for both tests.

Despite being harmful to the contaminated soil, the addition of rhamnolipid improved the bioremediation rates. The concentration of 4 mg g^{-1} of rhamnolipd reached 60% of TPH removal after 45 days of biodegradation assays.

One can conclude that at the beginning of the treatment, the addition of rhamnolipid had a negative impact on microbial activity and seed germination, although, during the treatment the soil microorganisms became more adapted to this addition, improving the biodegradation of the crude oil.

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REFERENCES

Alef K. and Nannipiere P. (1995) Methods in applied soil microbiology and biochemistry, London: Academic Press. 576p.

Banat I.M. (1995), Biosurfactants production and possible uses in microbial enhanced oil recovery and oil pollution remediation: A review, *Bioresource Technology*, **51**, 1-12.

Banks M.K. and Schultz K.E. (2005) Comparison of plants for germination toxicity test in petroleum contaminated soil, *Water, Air, and Soil Pollution*, **167**, 211–219.

Bitton G. and Koopman B. (1992) Bacterial and enzymatic bioassays for toxicity testing in the environment, *Rev. Environ. Contam. Toxicol*, **125**, 1-22.

Bognolo G. (1999) Biosurfactants as emulsifying agents for hydrocarbons, *Colloids and Surfaces*, **152**, 41-52.

Cubitto M.A., Móran A.C., Commendatore M., Chiarello M.N., Baldini M.D. and Sineriz F. (2005) Effects of Bacillus subtilis O9 biosurfactant on the bioremediation of crude oil-polluted soil, *Biodegradation*, **15**(5), 281-287.

Deschênes L., Lafrance P., Villeneuve J.P. and Samsom R. (1996) Adding sodium dodecyl sulfate and Pseudomonas aeruginosa UG2 biosurfactant inhibits polycyclic aromatic hydrocarbon biodegradation in a weathered creosote contaminated soil, *Applied Microbiology and Biotechnology*, **46**(5-6), 638-646.

Fletcher J. (1991) A brief overview of plant toxicity testing, In: Plants for toxicity Assessment, Goruch J.W., Lower W.R., Lewis M.A. and Wang W., ASTM, Philadelphia, PA.

Kapannen A. and Itavaara M. (2001) Ecotoxicity tests for compost applications, *Ecotoxicology and Environmental Safety*, **49**, 1-16.

Lin S-C. (1996) Biosurfactants:recent advances, *Journal chem. Tech. Biotechnol*, **66**, 109-120.

Millioli V.S., Santos L.C. dos, Rizzo A.C.L., Soriano A.U. and Santos R.L.C. (2005) Evaluation of optimum concentration of two anionic surfactant in the biodegradation of crude oil contaminated soil. 9th InternationalConference on Soil-Water Systems, France, 3 - 7 October.

Mueller J.G., Chapman P.J. and Pritchard P.H. (1989) Creosote contaminated sites, *Environ. Sci. Technol*, **23**, 1197–1201.

Mulligan C.N. (2005) Environmental applications for biosurfactants, *Environmental Pollution*, **133**, 183-198.

Nannipieri P., Grego S. and Ceccanti B. (1990) Ecological significance of the biological activity in soil, *Soil Biochem*, **6**, 293-355.

Nitschke M. and Pastore G.M., (2002), Biossurfactantes: propriedades e aplicações. *Quimica Nova*, **25**(5), 772-776.

Noordman W.H., Wacher J.J.J., Boer G.L. de and Janssen D.B. (2002) The enhancement by biosurfactants of hexacadene degrade by Pseudomonas aeruginosa varies with substrate availability, *Journal of Biotechnology*, **94**, 195-121.

Oblinger J.L. and Koburger J.A. (1975) Understanding and Teaching the Most Probable Number Technique, *Journal of Milk Food Technology*, **38**(9), 540-545.

OECD (Organization for Economic Cooperation and Development) (1984) Guideline 208: Terrestrial Plants, Growth Test, OECD Guidelines for testing of chemical, OECD, Paris.

Oleszczuk P. (2008) Phytotoxicity of municipal sewage sludge composts related to physico-chemical properties, PAHs and heavy metals, *Ecotoxicology and Environmental Safety*, **69**, 496–505.

Ratsep R. (1991) Biological variables for monitoring the effects of pollution in a small catchments areas. A Literature survey, *Environmental. Monitory. Nord*., **8**, 6-21.

Rojas-Avelizapa N.G., Rodriguez-Valquez R., Salval-Bohorquez S. and Alvarez P.J.J. (2000) Effect of C/N/P ratio and nonionic surfactant on polychlorinated biphenyl biodegradation, *World Journal of Microbiology and Biotechnology,* **16**(4), 319-324.

Vecchioli G.L., Del Panno M.T. and Painceira M.T. (1990) Use of selected autochthonous soil bacteria to enhance degradation of hydrocarbons in soil, *Environmental Pollution*, **67**, 249-258.

Volpon A.G.T., Vital R.L. and Casella A.R.C. (1998) Método NMP em microescala para contagem de microrganismos consumidores de hidrocarbonetos, Comunicação técnica SEBIO N. 06/98.

West C.C. and Harwell J.H. (1992) Surfactant and surface remediation, *Environm. Sci. Techonol.*, **26**, 2324-2330.

Zhou W., Zhu L. (2008) Enhanced soil flushing of phenanthrene by anionic and nonionic mixed surfactant, *Water Res*, **42**, 101-108.