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Phytoremediation using *Rizophora mangle* L. in mangrove sediments contaminated by persistent total petroleum hydrocarbons (TPH's)

Icaro T.A. Moreira [a,*], Olivia M.C. Oliveira [a,b], Jorge A. Triguis [a], Ana M.P. dos Santos [c], Antonio F.S. Queiroz [a], Cintia M.S. Martins [a], Carine S. Silva [a], Rosenaide S. Jesus [a]

[a] Núcleo de Estudos Ambientais, Instituto de Geociências, Universidade Federal da Bahia, Campus de Ondina, 40170-290, Salvador-BA, Brazil
[b] Instituto de Geociências, Departamento de Geofísica Aplicada, Universidade Federal da Bahia (UFBA), Campus de Ondina, 40170-290, Salvador-BA, Brazil
[c] Instituto de Química, Universidade Federal da Bahia (UFBA), Campus de Ondina, 40170-290, Salvador-BA, Brazil

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ABSTRACT

In this study developed a pilot-scale experiment during 0–3 months on the implementation of a Phytoremediation model with species *Rizophora mangle* L. and a model of Intrinsic Bioremediation, in order to try to compare which model would achieve the maximum effectiveness of degradation of total petroleum hydrocarbons in mangrove sediment. After 90 days a higher efficiency in removing organic compounds from sediment by Phytoremediation (87%) was observed. This larger efficiency in the remediation of the plant was enhanced with the largest growth of bacteria in its rhizosphere, reaching the highest CFU g^{-1}, 31×106. It was observed a larger growth of plants exposed to contaminated sediments (46.3 cm) compared to those grown in reference sediments (34.4 cm), suggesting a good adaptation. The data showed that the Phytoremediation is an effective in the degradation of TPH's, becoming a promising option in the application of the technique in mangrove areas.

1. Introduction

Accidents caused by oil spills have the potential to cause various environmental and economic effects on a wide variety of natural resources and services. Pilot studies based on environmental restoration of coastal regions are becoming increasingly necessary, given the importance of these ecosystems to the ecological balance and also because they are targets of major impacts of petrogenic origin, caused mainly by the oil industry accidents. The severity of these effects depends on the season, the discharge volume, type and location where such discharge occurs, and especially the environmental conditions at the time of occurrence [1,2]. Contamination of the aquatic environment has become a serious problem in many parts of the world, with rivers and bays often seriously affected. Almost all marine coastal ecosystems have complex structural and dynamic characteristics that can be easily modified by human influence. Estuarine and marine sediments are sinks for various contaminants transported from other ecosystems [3–7].

Total Petroleum Hydrocarbons (TPH's) represents one of the most common groups of persistent organic pollutants in the environment. They have been studied much more because they are toxic to many organisms and human health. The main sources of contamination in soil and sediment by TPH's include the different sectors of the petroleum industry, such as extraction, refining and consumption [8,9]. Remediating persistent TPHs from soils is generally a slow and expensive process. This is particularly true for the most recalcitrant portion of TPHs. For instance, the high molecular weight fractions derived from oil refinery sludge are exceptionally hard to remediate [8,16]. The process of TPH's removal in the sediments of aquatic environments is determined by its interaction with the system and controlled by physical and chemical factors, composition of the microbial community, the hydrodynamic site, sunshine, temperature, sediment grain size, nutrient availability, among others [10–13].

Many TPH's removal techniques in soils and sediments are being applied to attempt the restoration of environments, such as ex situ: Chemical Oxidation, Thermal Desorption, Biopiles and Incineration. Moreover, other in situ techniques, such as: Landfarming, Air sparging, Biosparging, Bioventing, Reactive Barriers, Bioremediation (Bioaugmentation and Biostimulation), Intrinsic Bioremediation (monitored natural attenuation) and Phytoremediation, were applied [10,14]. In recent years, there was a larger tendency for in situ methods once they offer less risk to the environment, which are efficient and cheap. With advances in biotechnology, Phytoremediation has emerged as the alternative that best fit the requirements listed here [15–18].

Phytoremediation is a biological technology that utilizes natural plant processes to enhance degradation and removal of contaminants in soil, sediments or groundwater. Broadly, Phytoremediation can be cost-effective in large areas with high residual-levels of contamination by organic, nutrient, or metal pollutants, when applied correctly [19]. The correct application depends on a previous study to be able to assess

* Corresponding author. Tel.: +55 71 3283 8632; fax: +55 71 3283 8632.
E-mail addresses: icaromoreira@ufba.br, icarotam@gmail.com (I.T.A. Moreira).

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the efficiency of the plant specimen to be applied and the possible risks to the ecosystems where it is applied.

In mangrove sediment, the capture, transformation, volatilization and rhizodegradation of TPH's are important processes that occur during Phytoremediation. Microbial degradation in the rhizosphere (rhizodegradation) may be the main mechanism for cleaning a variety of soils contaminated by petroleum, including mangrove sediments. This occurs because the contaminants, such as PAHs, are highly hydrophobic, and their absorption into the soil reduces their bioavailability for capture by plants and consequently their phytotransformation [19]. The success of rhizodegradation depends on the presence of and interaction between specific microorganisms, adequate environmental conditions and the oil availability [20].

A promising species for the application of Phytoremediation in mangrove sediments is *Rizophora mangle* L. (red mangrove), due to its characteristics of absorber plant, of strong interaction with the microbial community, not being sensitive to the presence of TPH's in the sediment [21,22]. *R. mangle* L. are native species found along the Atlantic coast from Florida to Southern Brazil, and in western Africa from Senegal to Angola. The cold climate in the North sets the limits of mangrove forests in the region. For the *R. mangle* in the U.S.A. coast is the limit, Bermuda the most precise. [23–26].

The objective in this study was to evaluate the efficiency of the *R. mangle* application to the Phytoremediation of contaminated sediments by TPH's. The research was based on a controlled pilot-scale, where it was the closest simulated environmental conditions of a mangrove. The sediment used was monitored for 90 days, with six samples, using physical, chemical and geochemical parameters and nutrients.

2. Materials and methods

2.1. Sediments sampling/collection and mixing

The sediments used in the models of remediation in this study were collected in an estuary located near the cities of Candeias and São Francisco do Conde, North of the Todos os Santos Bay, Bahia, Brazil. Sediment samples were collected from 0 to 30 cm depth at random from five locations. The sediment samples were sieved through a 4 mm sieve to eliminate coarse rock and plant material, thoroughly mixed to ensure uniformity. Five sub-samples were dried in a lyophilize cold for 72 h and sieved through 2 mm mesh to determine selected soil physical and chemical characteristics (Table 1). Particle-size distribution was determined after the organic matter was removed with 30% H_2O_2, by the [27] method. Soil organic matter was determined using a modified Mebius method [28]. Total N was determined by the Kjeldahl's digestion, distillation and titration method [29] and available P by the Olsen extraction method [30].

2.2. Addition of oil residual in the sediment

Sediment samples were mixed in a 1:10 ratio with oil residue found in the same area, a region with many activities in the petroleum industry (extraction, transportation and refining). Immediately after being mixed the oil residue with the sediment, five samples of the mixture were collected to analyze the concentration of TPH's. The composition of the oil residue used is shown in Fig. 1. It was collected a sediment in a reference area, as discussed in another research by [31] for comparisons of the parameters analyzed in this study.

Table 1
Some selected physicochemical properties of the sediment used in experiment.

Parameters	Value
Textural class	Sandy mud
Particle-size distribuition	
Sand (%)	23.65
Silt (%)	73
Clay (%)	3.25
Organic matter (%)	5.73
Organic carbon (%)	3.32
Total N (%)	0.36
Avaible P (mg/L)	1.8

2.3. Sediment remediation

All experiments were conducted in a greenhouse (Laboratory deployed to conduct research developed within the network RECUPETRO/UFBA — Cooperative Network Recovery in Areas Contaminated by Petroleum Activities, linked to the Federal University of Bahia) near the mangroves where they collected samples of sediments, in environmental conditions very close to the original ecosystem, with an average temperature of 24.6 °C. The dynamics of a mangrove was simulated, with tidal regime, sediment used for the application of remediation techniques. These simulation units were made of glass (50 × 30 × 40 cm). Within each unit of simulation 6 tubs of glass were added (30 × 10 × 10 cm) and they were applied to two models of remediation compared in this study. These tubs of glass, were suspended in the unit simulation, allowing the simulation of tidal regime with water runoff. Tubs of glass were closed at the bottom to prevent loss of chemical residue when watering. All units received the treatment simulation of daily tidal regime with an adequate amount of water (approximately 10 L) to maintain constant humidity of sediment, as in the mangrove ecosystem. The experimental projects are three replicates of each treatment and analysis of three samples from each repetition. To assess the efficiency of TPH's Phytoremediation in the sediment, each of the components described below were tested separately, taking into consideration public safety and local environmental compartments.

2.3.1. Phytoremediation

To evaluate the efficiency of Phytoremediation in mangrove sediment, the species *R. mangle* (red mangrove) was selected. This choice was based on pre-tests conducted earlier by our group as well as with other studies suggesting the use of this for Phytoremediation [22,31,32]. Seedlings of *R. mangle* were collected at low tide, taking into consideration their height (average of 3 months old), defining a standard sampling in order not to compromise the research results. The plants were submitted in sediments mixed with waste oil from the study area. In the laboratory simulation, the species were planted in glass tubes, where the daily regimen was simulated with the tidal water of the mangrove and morphophysiological monitoring was conducted during 90 days. During the growth period, plants were watered twice a week with bottled water as needed.

2.3.2. Bioremediation

It used the Intrinsic Bioremediation (Natural Attenuation Monitored) — where it was monitored with the degradation of hydrocarbons derived from petroleum hydrocarbon by bacteria present in the sediment mixed. It was characterized by the density of the bacterial community in order to compare the presence of microorganisms in Phytoremediation.

2.4. Quantification of bacterial community

For microbiological analysis, 25 g of different samples were transferred to Erlenmeyer flasks containing 90 mL of sterile 0.1% peptone water. Each sample was stirred at 200 rpm/30 min. For colony counting, it was used the technique of plating by “microgota” [33], decimal serial dilutions in agar nutrient agar (NA) (in g/ L beef extract, 3; bacteriological peptone, 5; NaCl, 3; agar, 13). The plates were incubated at 25 °C ± 1 °C for 24 h. After incubation, the plates selected were the ones that contained between 3 and 30 colonies. The

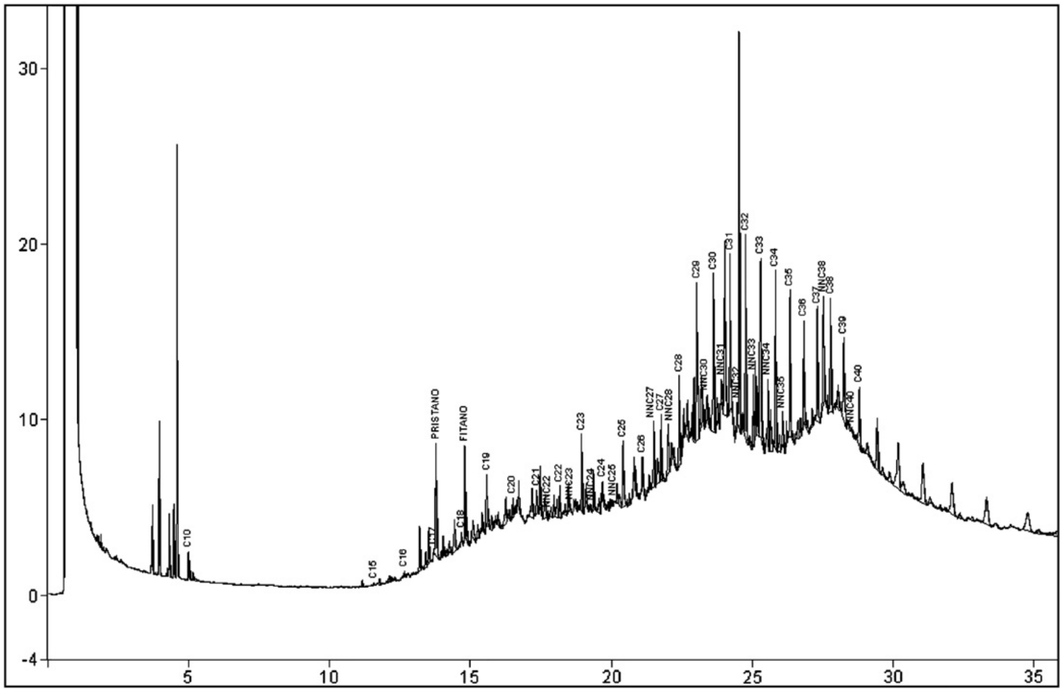

Fig. 1. Gas Chromatography (FID) of residual oil used in the study.

number of colonies counted was multiplied by the reciprocal of the dilution and the results expressed as Colony Forming Units (CFU). Quantification of bacterial density was assessed in two models of remediation.

2.5. TPH extraction and analysis

TPH levels in the sediment were determined by assaying for total hydrocarbons. Sediment samples (approximately 50 g) from their remediation experiments were collected at 0, 7, 15, 30, 60 and 90 days after the start of the experiments and were stored at 4 °C until analysis. The storage time for the collected samples was no longer than 10 days and the storage had no effect on TPH levels in soil (data not shown). The sediment samples were dried in a cold lyophilizer, constant temperature − 50 °C. The dried sediments (5 g) without previous treatment, were extracted with dichloromethane/hexane mixing (1:1, v/v). The extracts were concentrated to allow the solvent to evaporate completely, and then the amount of extracted sludge was determined gravimetrically. The extracted oil was weighed approximately 0.02 g for the fractionation of saturated compounds in an activated silica gel column and eluted with ultrapure hexane (30 mL). After that the eluted product was evaporated and then swelled to 1 mL with the same solvent elution. Extracts were quantified using a Varian CP 3800 gas chromatograph equipped with a DB-5 capillary column (30 m length, 0.25 mm ID, 0.25 lm film thickness) and Flame Ionization Detector (FID). GC conditions were as follows: injector temperature, 300 °C, starting oven temperature, 40 °C; 40 °C (hold 2 min) ramp 10 °C min^{-1} to 300 (hold 12 min); detector temperature, 300 °C. Helium was used as the carrier gas at a flow rate of 1.0 ml min^{-1} and a split ratio of 10:1 was used. Standard was prepared from the same TPH (C10–C40) stock chemicals.

2.6. Statistical analysis

It used analysis of variance in order to verify the existence or not of significant difference between the two models used. Whereas the condition to submit sample data to a parametric analysis of variance is that their variances do not show significant difference, it applied the test of Bartlett described in Beiguelman, to test the homogeneity of variances. To check the normality of data, the Kolmogorov–Smirnov test was applied. This test indicated, through a chi-square, that there is no significant difference between the variances of the samples. As variances were homogeneous, ANOVA was applied to a single parametric classification, which showed significant difference between the two models. But it has been done, “a posteriori”, a test for multiple parametric Turkey–Kramer to affirm the significant difference between the models. These statistical analyses were performed using the GraphPad Software.

3. Results

3.1. The effectiveness of the models remediation for removal of TPH's from sediment of mangrove

With the intention of evaluating the effectiveness of remediation models employed in this research (Intrinsic Bioremediation and Phytoremediation) for removal of HTP's in mangrove sediments contaminated, an experiment was conducted in pilot scale to compare the different methods of correction. The results showed that after 90 days the Intrinsic Bioremediation (Natural Attenuation Monitored) was able to remove 70% of TPH's individually, while the Phytoremediation (*R. mangle*) was able to remove approximately 87% of the TPH's present in the contaminated sediment (Fig. 2a). It was a statistically significant removal of the TPH's Phytoremediation with *R. mangle* regarding Intrinsic Bioremediation in contaminated sediments. These results indicate that the Phytoremediation with *R. mangle* has a larger capacity for degradation of TPH's in mangrove sediments. Analysis of TPH's removed by Phytoremediation with *R. mangle* showed that levels of contaminants in the sediment were reduced from 33.2 to 4.5 mg/g, while the Intrinsic Bioremediation has lowered from 33.2 to 9.2 mg/g in a growing season of 3 months (Fig. 2b). Thus, Phytoremediation was able to remove approximately 17% more sediment TPH's than the Intrinsic Bioremediation.

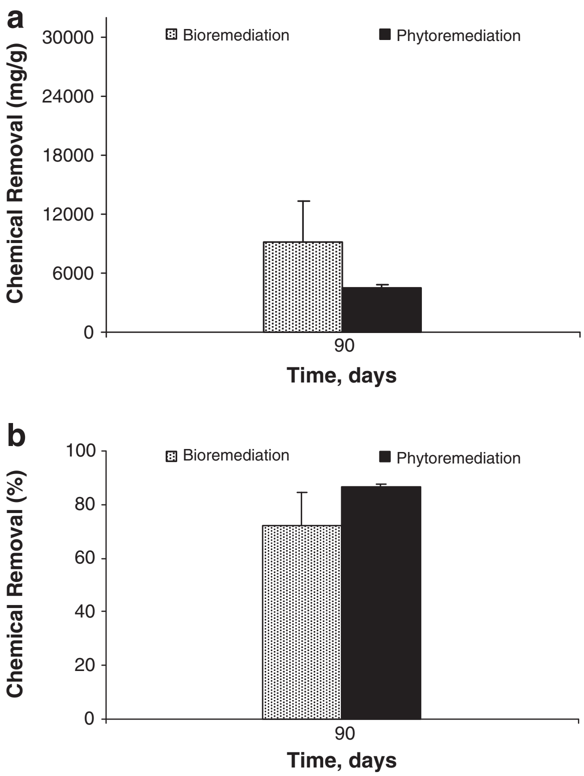

Fig. 2. TPH's removal for Intrisic Bioremediation and Phytoremediation (*Rizophora mangle* L.). The data are presented as percent of chemical removed relative to the sediment that contains 32.2 mg/g of 100% residual oil (n = 3). a) Indicates removal percentage, b) indicates the removal in mg/g.

3.2. The effectiveness of the models remediation for removal of different fractions of TPH's from sediment of mangrove

Based on Huang et al. [16], it was used fractions 3A (C16–23), 3B (C23–34) and 4 (C34–40) which are the most TPH's of recalcitrant contaminants in the sediment. These molecules are very resistant to remediation because of their fractions which are hydrophobic and have a high molecular weight. The results indicate that the Phytoremediation with *R. mangle* was more effective than the Intrinsic Bioremediation in the removal of all fractions of TPH's contaminated sediment. However in fraction 3A (C16–C23), both models remediation efficiencies gained quite close. In the fraction 3B (C23–34) the results showed that the degradation efficiency of Phytoremediation was moderately higher (82%) than that of Intrinsic Bioremediation (63%), while the fraction (C24–C40) this difference was the larger (Phytoremediation: Intrinsic Bioremediation and 70%: 21%). After 3 months of the Phytoremediation with *R. mangle* had fallen into major components of fractions 3A, 3B and 4, with an efficiency of about 78%, the Intrinsic Bioremediation declined only about 55%, taking into account the levels of total remediation TPH's (Fig. 3).

3.3. Temporal analysis of the models remediation for removal of TPH's from sediment of mangrove

It was assessed the effectiveness of two models of remediation applied (Phytoremediation and Bioremediation Intrinsic) based on the total content of TPH's staying in the mangrove sediment a function of time (Fig. 4). The repair rate remained relatively constant for Phytoremediation, resulting in pseudo-zero order kinetics for the whole period of 3 months. This behavior of Phytoremediation became a more effective model than the Intrinsic Bioremediation, despite having degraded a higher rate at the beginning of the experiment, failed to keep their initial rates of recovery during the experiment. After 90 days, the total amount removed by TPH's *R. mangle* was approximately 87%, while for the bioremediation was approximately 70%, with a strong decrease in the rate of removal.

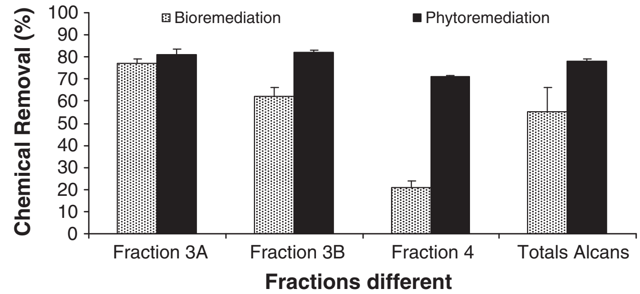

Fig. 3. Chemical removal (%) the fractions different in the remediation models, after 90 days (n = 3).

3.4. Temporal quantification of bacterial community of the models remediation

During the 90 days of the experiment, the total number of viable bacteria for the two models remediation (Phytoremediation and Bioremediation Intrinsic) applied to sediment contaminated with TPH's, were quantified in six pre-established samples. The results concerning the initial average count of bacteria are between 0.1 and $0.2\times10^6\times10^6$ CFU g^{-1}, determined at the beginning of the experiment. After being applied the models of the remediation in sediments, there was a significant increase in the number of microorganisms after the 7th day in the two models, showing significant difference compared to the initial sediment sample, 8.3×106 and 8.8×106 CFU g^{-1} respectively. After the 30th day there was a drastic drop in the number of microorganisms in the application model of Intrinsic Bioremediation (1.8×10^6 CFU g^{-1}), however there was an increase in Phytoremediation of the microbial community, and quantified values from 20.2×10^6

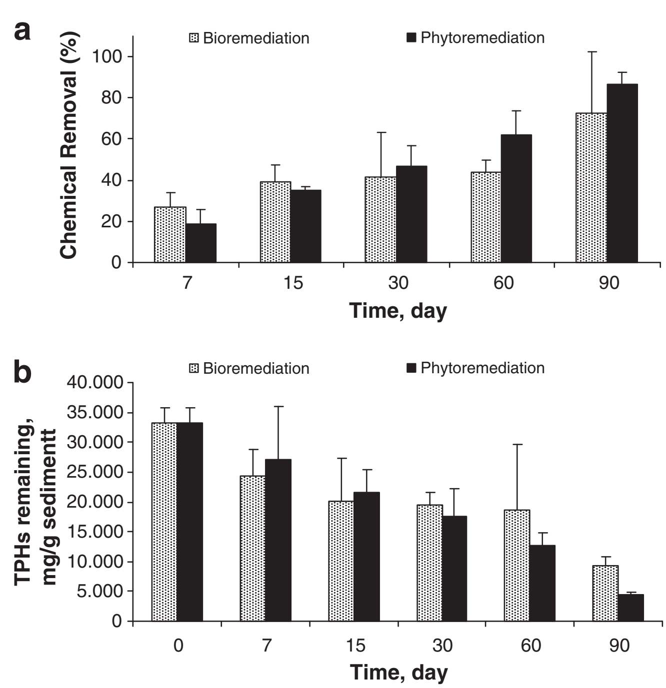

Fig. 4. TPH's removal in the mangrove sediment a function of time (n = 3). a) Indicates removal percentage, b) indicates the removal in mg/g.

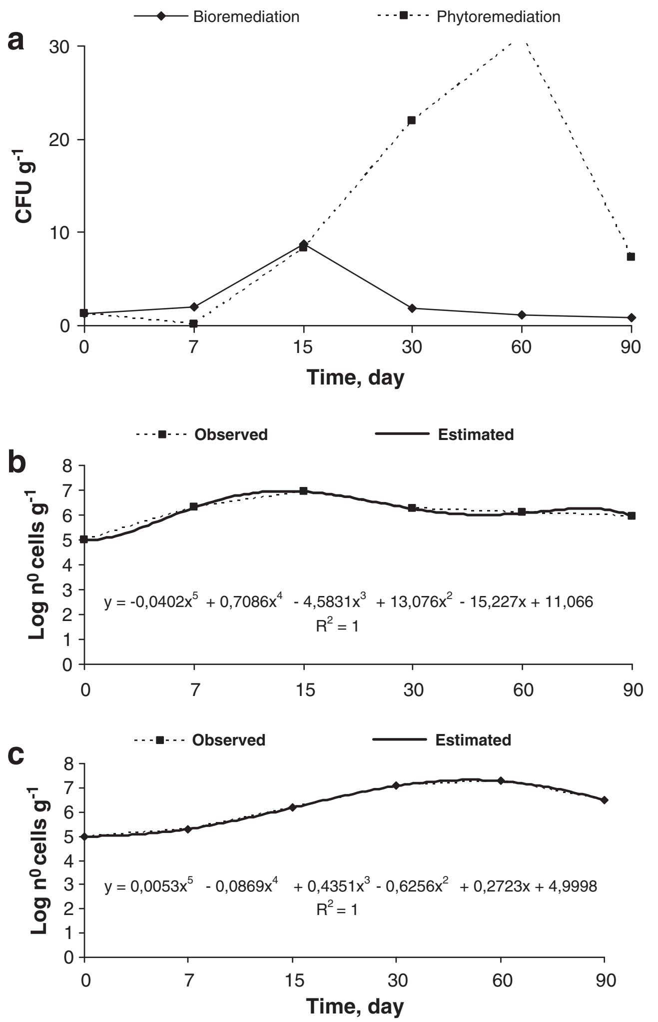

Fig. 5. Total count of bacteria during 90 days, with data expressed in polynomial trend with a coefficient of determination R^2 of 100% (n = 3). a) Comparison between models, b) count of bacteria in the Intrinsic Bioremediation, observed and estimated, c) count of bacteria in the Phytoremediation, observed and estimated.

to 24.4×10^6 CFU g^{-1}. Fig. 5 presents the total count of bacteria for 90 days, with data expressed in polynomial trend with a coefficient of determination R^2 of 100% for total bacterial counts.

3.5. *Physiology of Rizophora mangle used for Phytoremediation*

The sediments have high concentrations of TPH's, like those that were quantified in this study are quite toxic to plants, as these contaminants cause in many cases a negative impact on the vegetation growth of plants. Thus, the effects of TPH's in the sediment on the growth of *R. mangle* were evaluated by measuring the sizes of plants and their roots, by comparing the growth of plants of the contaminated sediment with the sediment reference (Fig. 6). Unexpectedly there was a higher growth in the experiments of Phytoremediation in contaminated sediments compared to the reference sediment, watching 22% increases in plant growth and root 4% bigger. Therefore, biomass accumulation of plants in contaminated sediment was higher than the plants in sediment reference non-contaminated. This growth increased in the red mangrove sediments probably indicates that the plant has a good adaptation to the conditions found in contaminated sediment, allowing for larger growth than plants in contaminated sediment.

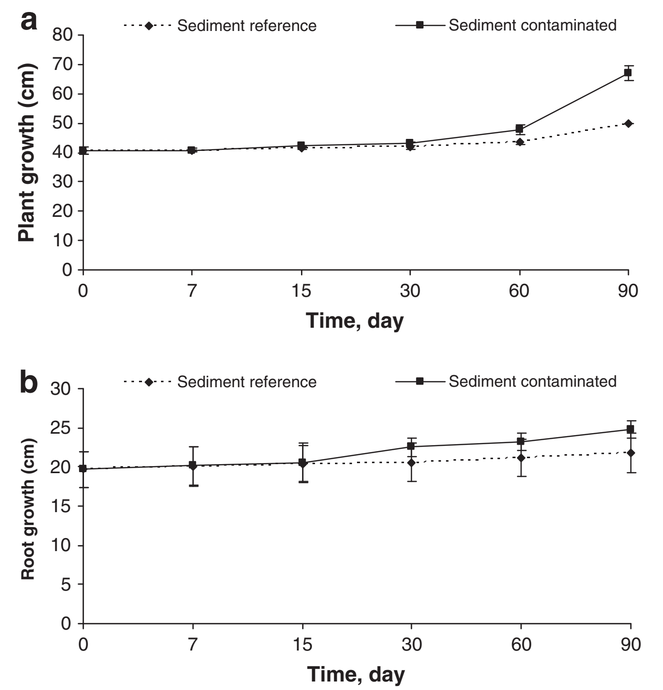

Fig. 6. Growth of *R. mangle* evaluated by measuring the sizes of plants (a) and their roots (b) (n = 3).

4. Discussions

The results of this study showed that the model of Phytoremediation with *R. mangle* is more effective in removing each the TPH's fractions in the contaminated sediment in relation to Intrinsic Bioremediation. Despite this trend already observed in other studies [34–36] of remediation processes applied in sediments contaminated by TPH's, it is still observed an increased use of other techniques in the recovery of areas impacted by oil activities, such as the “Land farming” [17,37,38] that is a bioremediation technique in field scale at which the surface of contaminated sediments are removed by wind, enhancing the activity of endogenous microorganisms and Intrinsic Bioremediation. However, these conventional techniques mentioned, although having a larger number of applications, especially in industrial areas contaminated, they have severe limitations in the removal of highly hydrophobic organic compounds, problems of degradation in sediments that have concentrations of contaminants at different heterogeneous depths, mainly when applied individually in the contaminated areas. This research shows once again that the Intrinsic Bioremediation is less efficient in the degradation of TPH's than the Phytoremediation.

The results for the removal of different fractions of TPH's found in this study showed that application of Phytoremediation was more efficient for the three fractions analyzed – 3A (C16–23), 3B (C23–34) and 4 (C34–40) – after the 90 days experiment compared with Intrinsic Bioremediation. This is justified probably because of the Phytoremediation act in the removal of contaminants jointly with different processes which also includes transfer, stabilization and destruction of organic compounds in sediments [39,40]. Degradation mechanisms that may have used *R. mangle*, ranges from phytostabilization, preventing the absorption and acts by phytostimulation of microorganisms present in its rhizosphere, therefore acting with rhizodegradation. One should also consider the possibility of the plant had absorbed the organic compounds and, later had achieved the phytodegradation. However, despite these possibilities, probably the mechanisms used by the red mangrove, had the lowest efficiency for fraction 4 (C34–40), which was observed in the results, although quite significant compared with Intrinsic Bioremediation, which had a decrease in kinetic remediation after the degradation of the lighter compounds. This is because of the Phytoremediation which is a set of processes acting in the degradation of organic compounds, unlike the Natural Attenuation.

The total number of bacteria that degrade hydrocarbons was evaluated during the application of two models of remediation over 90 days of the experiment, where the concentration in Intrinsic Bioremediation was higher until day 30, compared to Phytoremediation, hence indirectly the most initial efficiency degradation of different fractions of organic compounds. However, from the 30th day on it was observed an increase in bacterial density in sediment treated by red mangrove, reaching a count up to ten times more than the Bioremediation, which in turn may have caused a major acceleration in the kinetics of remediation and with it a more efficient process. The vegetated sediment microbial community is usually larger than that of non-vegetated sediment [41]. Importantly, the presence of contaminants and root exudates usually modifies the composition and activity of these communities [15,42]. This higher concentration of bacteria in contaminated sediments, was also evidenced by other researchers in studies that evaluated the degradation of organic compounds [15,43], confirming that the growth of hydrocarbon degraders was favored by the presence of the plant.

Importantly, the actuation of the rhizosphere on the degradation of contaminants already well reported in surveys [44]. In the case of *R. mangle,* this plant should probably produce allelopathic compounds, similar to organic compounds that stimulate the defenses of the communities of microorganisms in the face of environmental stress conditions, besides the possibility of entry of oxygen made possible by the rhizosphere [45,46]. Other studies evaluating the degradation of toxic compounds also found the presence of compounds exuded by the roots, such as carbohydrates, organic acids and amino acids that probably might have stimulated the degradation of contaminants [47].

5. Conclusions

The study results in a pilot scale showed that the model applied to Phytoremediation with *Rizophora mangle* achieved larger efficiency in the degradation of different fractions of TPH's, reaffirming this technique to be promising in the recovery of areas contaminated by the activities of the oil industry, in addition to be an environmentally correct technique. The study found that the monitored natural attenuation (intrinsic bioremediation) has low efficacy when applied individually, although initially it has been more effective in the degradation of contaminants. Moreover, the data of microbiological analysis found that the association of plants with the community of microorganisms in the rhizosphere enhanced the degradation of organic compounds in the sediment, with 87% efficiency, and foster increased growth of these plants. It is suggested that a more detailed study would combine these processes into a new product for application in remediation of contaminated sediments by mangrove TPH's, especially when dealing with sediment contamination heterogeneous at different depths. New researches on the transformation of TPH's in the environment are needed to see whether this transformation produces toxic co-products. Finally, it is important to assess whether the model of Phytoremediation produced in pilot scale in this study is as effective in situ, large scale, as it was observed under laboratory conditions.

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