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Natural attenuation of weathered oil using aquatic plants in a farm in Southeast Mexico

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ABSTRACT

An experiment was conducted in field for three years to assess the sustainability of aquatic plants *Leersia hexandra*, *Cyperus articulatus,* and *Eleocharis palustris* for use in the removal of total hydrocarbons of weathered oil in four areas contaminated with 60916–119373 mg/kg of hydrocarbons. The variables evaluated were coverage of plant, dry matter, density of plant growth-promoting rhizobacteria, and the removal of total weathered oil. The variables showed statistical differences ($p = 0.05$) due to the effects of time and the amount of oil in the soil. The three aquatic plants survived on the farm during the 36-month evaluation. The grass *L. hexandra* yielded the greatest coverage of plant but was inhibited by the toxicity of the oil, which, in contrast, stimulated the coverage of *C. articulatus*. The rhizosphere of *L. hexandra* in control soil was more densely colonized by N-fixing bacteria, while the density of phosphate and potassium solubilizing rhizobacteria was stimulated by exposure to oil. *C. articulatus* coverage showed positive relationship with the removal of weathered oil; positive effect between rhizosphere and *L. hexandra* grass coverage was also identified. These results contributed to the removal of weathered oil in Gleysols flooded and affected by chronic discharges of crude oil.

KEYWORDS
Cyperus; *Eleocharis*; *Leersia*; phytoremediation; rhizobacteria

Introduction

Oil is the main source of energy, accounting for 36.7% of the world consumption of energy, while natural gas accounts for 29.6%; both cover 66.3% of the energy demand in the world (BP 2014). Oil use contributes to the development of the economy (White *et al.* 2006; Mohsenzade *et al.* 2009; Pérez-Hernández *et al.* 2013; BP 2014), but also causes environmental problems and harm to living beings through the contamination of soil, water, and sediments with hydrocarbons (Muratova *et al.* 2008; Mohsenzade *et al.* 2009). In southeast Mexico, cattle farms located in wetlands surrounding oil infrastructure are examples of the environmental impact of oil. These farms have been replanted with grass and sedges adapted to the anaerobic conditions and the toxic effects of weathered oil (Rivera-Cruz 2011a, b). Natural attenuation processes take place in oil-contaminated sites; it is a technique of bioremediation *in situ* that reduces the concentration of oil and its derivatives. Dilution, dispersion, volatilization, adsorption, and biodegradation are examples of natural attenuation in the environment (Lee *et al.* 2001). Natural attenuation is effective for cleaning groundwater (Leibenath *et al.* 2006) and flooded soil, involving the use of native plants that can decontaminate contaminated soil. This technique is called phytoremediation (Cunningham *et al.* 1996), or using plants to decontaminate water and soils (Merkl *et al.* 2005; Maldonado-Chávez *et al.* 2010; Liao *et al.* 2015). Plants have the advantage of carrying out chemical reactions using sunlight to metabolize or mineralize organic molecules (Olguín *et al.* 2007); they create the rhizosphere, formed by roots, microorganisms, and associated enzymes that absorb, transform, degrade or stabilize soil contaminants (Cunningham *et al.* 1996). The rhizosphere is the habitat of plant growth-promoting rhizobacteria (PGPR), which, in addition to releasing nutrients to the soil solution, can develop the ability to use hydrocarbons as a source of carbon and energy (Cunningham *et al.* 1996). The functions of the group of PGPR bacteria are to fix atmospheric nitrogen, solubilize nutrients (phosphate and potassium), and synthesize plant hormones, making the soil more fertile and of better quality for other organisms (McGrath *et al.* 2001; Vessey 2003), which in turn improves the provision of environmental services. The removal of contaminants from the soil by phytoremediation has been demonstrated in several studies (Kaimi *et al.* 2006; Robertson *et al.* 2007; Razmjoo and Adavi 2012; Liao *et al.* 2015). The fasciculated (fibrous) roots of grasses create an extended rhizosphere with potential to host PGPR and with tolerance to recalcitrant hydrocarbons (Merkl *et al.* 2005; Maldonado-Chávez *et al.* 2010). The participation

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of PGPR in detoxification, mineralization, and humification of weathered oil has not yet been tested *in situ*, though several authors (Pothuluri and Cerniglia 1994; Chaudhry *et al.* 2005) suggest that the bacteria *Pseudomonas putida*, *Mycobacterium sp.*, and *Rhodococcus sp.* perform this function through the hydroxylation of benzene rings. This study evaluated the effect, under *in situ* conditions, of the herbaceous plants *Cyperus articulatus* L., *Eleocharis palustris* (L.) Roem. & Schult., and *Leersia hexandra* Swartz on the attenuation of a chronic spill of crude oil in southeast Mexico. The grass *L. hexandra* is used to feed cattle and goats, while *C. articulatus* and *E. palustris* are hosts to aquatic fauna and invasive species in floodplains of tropical regions used for cattle grazing (Novelo 2006). The objectives of this study were to evaluate the sustainability of *C. articulatus*, *E. palustris,* and *L. hexandra* for use in creating vegetation cover, hosting rhizobacteria, and removing weathered oil in four areas of a farm contaminated with weathered oil. This will allow us to know the natural attenuation potential of these plant species in the southeast of Mexico. We propose a hypothesis stating that different concentrations of weathered oil induce differences in the growth and cover of aquatic plants, and that they increase the density of rhizobacteria belonging to the groups N-fixing bacteria (NFB), phosphate solubilizing bacteria (PSB), and potassium solubilizing bacteria (KSB). The hypothesis also states that the three aquatic plants and the rhizobacteria are adapted to anaerobic soils and that they can improve the removal of weathered oil for natural attenuation.

Materials and methods

Study area and environmental conditions

The study was conducted over 36 months (2010–2013) in a farm of 2.43 ha in the ejido Jose Narciso Rovirosa, Huimanguillo, Tabasco, located in southeast Mexico (18° 07' 37" N and 94° 04' 28" W) at 17 m.a.s.l. (Figure 1). The climate is classified as Amf (A, tropical clime; m, monsoon rains; and f, with almost constant rainfall), with an average annual rainfall of 2200 mm, 1200 mm of evaporation and a mean annual temperature of 26°C (INEGI 2001;CONAGUA 2014). The farm is part of a coastal plain dominated by wetlands with gleyed soils flooded up to 0.9 m deep, used for forage. The vegetation consists of a meadow with grasses and rooted aquatic weeds. On the surface, there are pipelines of 4, 6, and 24 inches in diameter; underground, at less than one meter in depth, there are other pipelines that transport crude oil and natural gas to the Gas Petrochemical Complex of La Venta (CPGLV by its acronym in Spanish). There is also a capped oil well and a waste management dam.

Oil spill in the farm

The accumulation of crude oil in the soil was caused by the rupture of oil pipelines connecting oil wells with CPGLV and by the leakage of drilling mud from storage dams of nearby

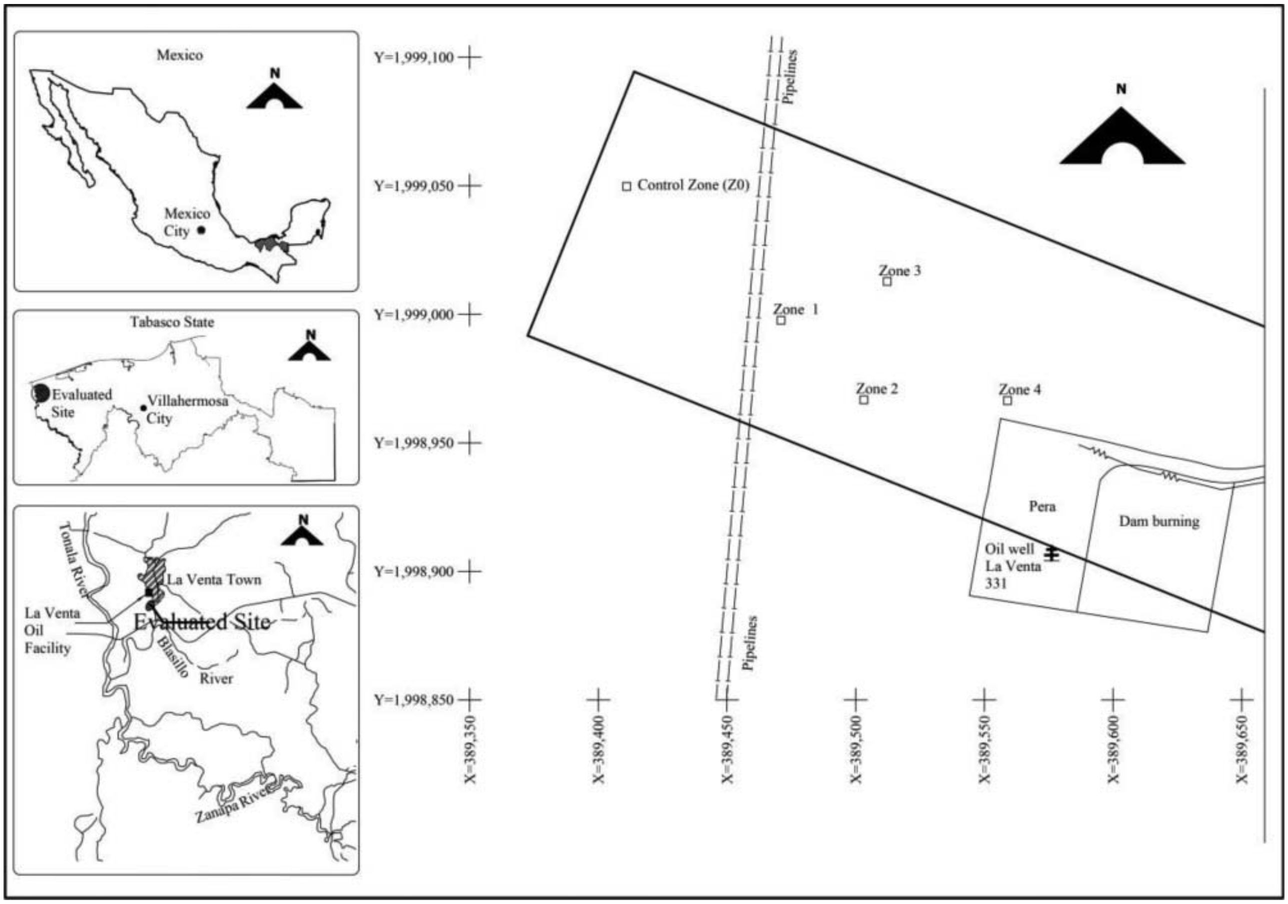

Figure 1. Localization of study site.

wells. The spill of oil has been chronic since the establishment of the oil complex (PEMEX 1988), and the spilled oil has been exposed for 50 years to physical, chemical, and biological environmental factors. The amounts of total weathered oil in the soil surface layer (0–50 cm) range from 968 to 495515 mg/kg of dry soil (Carranza 2011).

Collection of soil, oil, and variables studied

The geographical location of the five zones studied is reported in Table 1 and Figure 1. Each zone (Z) consisted of an area of 16 m^2 (4 × 4 m) (Figure 2). Within each Z, we cut and pulled the plant biomass, then removed, fractionated and homogenized the soil. Within the 9 m^2 at the center of each Z, we delimited 144 pseudo-plots (PSE) of 0.25 × 0.25 m, with an edge effect of 0.50 m on each side; then, we randomly collected soil from 30% of the surface of the PSE to assess the initial concentration of total weathered oil by Soxhlet with dichloromethane (99.5%) (EPA 1996) and by gravimetric quantification (DOF 2006). The initial concentrations of total weathered oil in zones 1–4 were high, while in control soil (Z0) they were low (Table 1), according to the allowable values of the heavy fraction specified by NOM-138-SEMARNAT/SA1–2008 (DOF 2010). From a sample composed of 43 sub-samples (30% of PSE) it was prepared and used to determine the texture class. The texture of the five areas is loam (Bouyoucos 1962), the pH, the soil organic carbon (Walkley and Black 1934), the total nitrogen by micro-Kjeldahl digestion with H_2SO_4 (Page *et al.* 1982), and the available phosphorus (Olsen and Sommers 1982).

Plant species used in the study

L. hexandra, *E. palustris,* and *C. articulatus* were used in this study; all are dominant plants in the farm and provide environmental services throughout the year. *L. hexandra* is a perennial forage grass that creates a rhizosphere with its branched, fibrous, and stoloniferous roots. The branches are semidecumbent, and the stems are flexible with narrow and small leaves. The foliage forms a cushion on the water and soil, locally known as "tembladera." As for *E. palustris* (tulillo) and *C. articulatus* (tule), they are wild plants of the Cyperaceae family that are hosts to aquatic fauna. *E. palustris* has a triangular stem (Asturnatura 2013). *C. articulatus* has a circular stem, septate, ascending, of up to 4 cm or more in height from an underground, hard, and scaly rhizome (Novelo 2006).

Establishment of the experiment

The experimental zones were planted with budding stems of *L. hexandra* and rhizomes of *E. palustris* and C. *articulates* collected from adjacent noncontaminated areas (100 of each species per zone, equally distributed). Each Z was fenced with a galvanized wire mesh 1.5 m high to keep away grazing cattle during the 36 months of the evaluation (Figure 2).

Coverage of plant, dry matter, bacteria density, and removal weathered oil

At months 12 (T1), 24 (T2), and 36 (T3), corresponding to May 2011, 2012, and 2013, variable coverage of the plant (%), dry matter (g/m^2), density of bacteria (NFB, PSB, and KSB; CFU/g/dry soil), and removal of weathered oil (%) were evaluated.

Coverage of plant and dry matter

At 12, 24, and 36 months, we collected the plant biomass at ground level of *L. hexandra*, *E. palustris,* and *C. articulatus* from plots of 1m^2 within each of the five Z, in tetraplicate. The biomass was placed in paper bags and dried in oven at 72°C for 72 hours. The vegetation cover each species was evaluated before cutting the biomass, using the following equation: $\text{Coverage}\ (\%) = \text{Nc} * \frac{100}{\text{n}}$, where Nc is the number of the plant species in the pseudo-plot (1 × 1 m) and n is the total number of species within the m^2 (Stiling 1999).

Density of rhizobacteria

In each pseudo-plot four rhizospheres per plant species were collected and the CFU of NFB, PSB, and KSB were evaluated. The specific culture media were combined carbon for free-living NFB (Rennie 1981), Pikovskaya for PSB (Pikovskaya 1948), and according to Qi-Mei *et al.* (2002) for KSB. It used 10 g of fresh soil and the technique of serial dilution (10^{-1} to 10^{-5}) on agar plate (Madigan *et al.* 2009).

Removal of hydrocarbons of weathered oil

The natural attenuation was measured using the percentage of removal of total weathered oil in mg/kg dry basis. At 12, 24, and 36 months, soil was collected from each Z of the 43 pseudo-plots and total weathered oil was extracted (mg/kg, dry basis) for 8 hours using Soxhlet equipment with dichloromethane (99.5% purity). The removal of oil was calculated by the difference between the initial concentration of total weathered

Table 1. Geographical location and chemical properties of the soil (May, 2010).

	UTM Coordinates		pH	(%)		(mg/kg dry basis)	
Zone	X	Y	(H_2O, 1:2)	SOC†	N_{total}	P_{avail}	Total oil
Z0	389416.62	1999049.7	4.3	14.1	0.28	11	968 ± 356
Z1	389471.02	1998997.6	4.16	9.3	0.13	13.6	60916 ± 15202
Z2	389503.18	1998966.7	4.21	12.8	0.21	5.8	76086 ± 4690
Z3	389512.26	1999012.7	3.83	13.4	0.2	9.4	92096 ± 20300
Z4	389559.04	1998966.4	4.16	9.02	0.20	6	119373 ± 28764

†SOC: Soil organic carbon.

Figure 2. Experimental plot in control zone. In front the Japanese grass (*Leersia hexandra*), and cattail closed bottom core (*Typha sp.*).

oil and the final concentration recorded at 12, 24, and 36 months, multiplied by 100.

Statistical analysis

Bifactorial ANOVA was used to analyze the data of coverage of plant, dry matter, CFU of rhizobacteria (NFB, PSB, and KSB), total weathered oil, and removal of total weathered oil, considering the concentration factors of total weathered oil and time. The differences between the variables were assessed using Tukey's mean separation test ($p = 0.05$), and the homogeneity of the variation in the data was verified. The analysis was performed using the statistical program SAS v.8.01 (SAS 2005).

Results

Coverage of plant

The means of the coverage of *L. hexandra*, *E. palustris,* and *C. articulatus* in the four contaminated Z showed statistically significant differences ($p = 0.05$) at 12, 24, and 36 months compared to the control value (Z0) (Table 2). The grass *L. hexandra* had the most extensive coverage in the control soil, with 100% at 12, 24, and 36 months, but decreased with increasing amounts of total weathered oil in the soil of the four contaminated Z. The exposure of *L. hexandra* to 119373 mg/kg of weathered oil for 12 months inhibited its coverage to 9.4%; however, the toxic effect of 76086 mg/kg was minimal, with a coverage of plant of 95.7%, very close to the value of Z0 (100%). The grass cover was less inhibited at 24 and 36 months, with significant statistical differences ($p = 0.05$) compared to the cover at 12 months (Table 2). The coverage of the sedges *E. palustris* and *C. articulatus* at 36 months showed a positive trend with increasing concentrations of total weathered oil; the correlation values were 0.695^{*} and 0.768^{**}, respectively.

Dry matter

The dry matter produced by *L. hexandra*, *E. palustris,* and *C. articulatus* showed significant statistical differences ($p = 0.05$) due to the effect of time, total weathered oil doses, and their interactions (Tables 2 and 3). The grass *L. hexandra* accumulated the greatest amount of dry matter at 12, 24, and 36 months in both the control soil (Z0) and in the four soils affected by total weathered oil (Table 2); likewise, the amount of dry matter decreased due to the toxic effect of the presence of high levels of oil in the soil. The largest amount of dry matter at 12 months was 502 g in the control soil (Z0), which decreased to 4.9 g in soil with 119373 mg/kg, a decrease of 10200%. The amount of dry matter of *L. hexandra* recorded at 12 and 36 months showed a negative correlation (-0.921^{**} and -0.775^{**}) with the amount of total weathered oil. As for the production of dry matter by *E. palustris*, it increased at 36 months with the increase of oil in the soil, and the correlation was 0.695^{*}; in contrast, the dry matter of *C. articulatus* decreased, showing a correlation of -0.845^{**} (Table 2).

Density of rhizobacteria

The effects on the means of the three groups of rhizobacteria exerted by the factors of time of exposure, concentration of total weathered oil, and interactions between factors were significant ($p = 0.05$) (Tables 3 and 4). At 12, 24, and 36 months, the means of the densities of CFU of NFB, PSB, and KSB isolated from the rhizosphere of three aquatic plants, showed statistical

Table 2. Changes in plant coverage and dry matter due to the effect of different concentrations of total weathered oil and to the time of exposure.

	T1[†] (Month 12)			T2 (Month 24)			T3 (Month 36)		
Total oil (mg/kg)	Lh	Ep	Ca	Lh	Ep	Ca	Lh	Ep	Ca
					Coverage (%)				
Z0: 968	100a[†]	0e	0d	100a	0b	0b	100a	0c	0c
Z1: 60916	62.8c	9c	28.2b	100a	0b	0b	70c	0.4c	29.6a
Z2: 76086	95.7b	4.3d	0d	100a	0b	0b	98.5b	1.5c	0c
Z3: 92096	51.5d	6.7cd	41.8a	84.18c	15.2a	0.62b	74.5c	10b	15.5b
Z4: 119373	9.4e	77a	13.5c	95.3b	0.4b	4.3a	79.9c	14.9a	5.2c
					Dry matter (g m^{-2})				
Z0: 968	502a	0e	0d	483.9a	0c	0c	615a	0e	0c
Z1:60916	201.4d	9c	90.2b	290.7c	0c	0c	453c	2.4d	192a
Z2:76086	401.4b	4.3d	0d	261.6e	0c	0c	565b	8.6c	0c
Z3:92096	344.6c	6.7cd	279.8a	270.5c	49a	2b	300.5d	40.6b	62.4b
Z4:119373	4.9e	77a	7c	320.9b	1.3b	14.4a	240e	45a	15.2c

T[†] = time; Lh = *Leersia hexandra*; Ep = *Eleocharis palustris*; Ca = *Cyperus articulatues*; Z = Zone. [†]Different letters within each column indicate statistical differences (Tukey, p = 0.05 and n = 4).

Table 3. Significance test of time (T), concentration (C) of total weathered oil in soil, and interaction between the chemical and bacteriological variables of the plants.

Variable	Time (T)	Total oil (C)	Interaction (T)(C)	CV (%)
Total weathered oil[†] (mg/kg dry soil)	<0.001	<0.001	<0.001	18.75
Removal of total weathered oil (%)	<0.001	<0.001	<0.001	11.75
Dry matter [*Leersia hexandra* (Lh)] (g/m^2)	<0.001	<0.001	<0.001	10.11
Dry matter [*Eleocharis palustris* (Ep)] (g/m^2)	<0.001	<0.001	<0.001	0.705
Dry matter [*Cyperus articulatus* (Ca)] (g/m^2)	<0.001	<0.001	<0.001	12.07
Total dry matter (Σ= Lh+Ep+Ca) (g/m^2)	<0.001	<0.001	<0.001	18.29
[†]NFB (Lh+Ep+Ca) (CFU/g soil)	<0.001	<0.001	<0.001	89.02
PSB (Lh+Ep+Ca) (CFU/g soil)	<0.001	<0.001	<0.001	70.45
KSB (Lh+Ep+Ca) (CFU/g soil)	<0.001	<0.001	<0.001	64.01

[†] NFB, free- living N-fixing bacteria; PSB, PO_4-solubilizing bacteria; KSB, K-solubilizing bacteria.

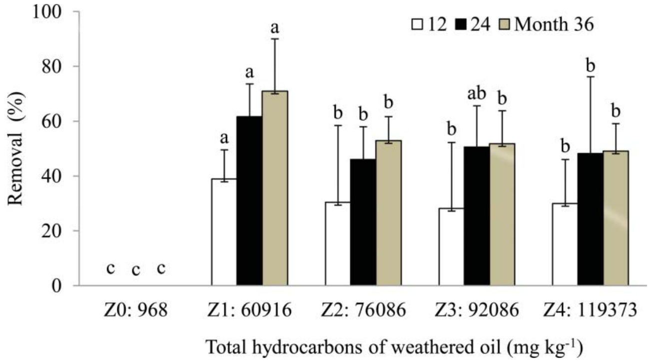

Figure 3. Removal of hydrocarbons of weathered oil per zone (Z) at three different times. Different letters within each column indicate statistical differences (Tukey, p = 0.05, n = 4).

differences ($p = 0.05$); a similar effect was caused by the amount of total weathered oil in the five Z of the farm (Table 4).

When comparing the densities of CFU of NFB, PSB, and KSB at 12, 24, and 36 months, we found that the rhizosphere of *L. hexandra* was more colonized at the three times evaluated. The highest density was 1490 × 10^3 CFU at 36 months; by contrast, the density was lower in the rhizosphere of the sedges *E. palustris* and *C. articulatus*, which showed no evidence of a clear trend over time (Table 4). The correlation of rhizobacteria with the amount of total weathered oil during Month 12 was positive and highly significant in the case of KSB isolated from the rhizosphere of *C. articulatus* and *E. palustris* (0.920* and 0.624**); during the Month 24, a positive correlation was shown by the PSB of *L. hexandra* and the PSB and KSB of *E. palustris* (0.692**, 0.725**, and 0.627**). The results for the Month 36 showed that the mean of NFB of *L. hexandra* was highly significant (0.756**), as were the means of the PSB and KSB of *E. palustris* (0.870** and 0.667*), and the KSB isolated from *C. articulatus* (0.623*).

Removal of weathered oil

The removal of total weathered oil in the four contaminated Z showed significant statistical differences ($p = 0.05$) in each of the three times evaluated (Figure 3). The highest level of removal occurred in Z1, which had an initial concentration of 60916 mg/kg that decreased 38.9, 61.6, and 71% at 12, 24, and 36 months, respectively. The lowest rate of removal was in Z4, which had an initial concentration of 119373 mg/kg that decreased 30, 48.2, and 49.1% at 12, 24, and 36 months, respectively. The ratio of removal with the concentration of total weathered oil at Month 12 was highly significant and negative (−0.629**); the same response occurred at 24 and 36 months (−0.958** and −0.862**). The results of CFU counted at Month 12 did not show any relationship between removal of oil and the three groups of rhizobacteria; at Month 24, there was a negative relationship between removal and the density of the PSB of *L. hexandra* (−0.760**), as well as with the density of the PSB present in the rhizosphere of *C. articulatus* (−0.659*). The results at 36 months were highly significant, with negative relationship between densities removal weathered oil with BSP and BSK *C. articulatus* (−0.812** and −0.843**), plus positive relationship (0.714**) with BSP of *E. palustris*. The data from Month 12 showed that the relationship between removal of oil and dry matter of *L. hexandra* was negative and significant (−0.613*), as was also between removal and the coverage of *C. articulatus* (−0.733**); in contrast, the relationship was positive (0.920**) with the coverage of *C. articulatus* at Month 36. Finally, removal of total weathered oil showed a negative relationship (−0.751**) with the coverage of *L. hexandra*.

Discussion

This research is one of the first studies showing the importance of native aquatic plants, in combination with their

Table 4. Densities of growth-promoting bacteria in the rhizosphere of three aquatic plants exposed to different concentrations of weathered oil for 36 months.

	NFB			PSB			KSB		
Variable	[†]Lh	Ep	Ca	Lh	Ep	Ca	Lh	Ep	Ca
Time (Month)				10^3 CFU (g^{-1} dry rhizosphere)					
T1 (12)	710ab[††]	101a	24a	148b	94a	10a	120b	33a	44b
T2 (24)	901b	333a	25a	151b	137a	7b	1139b	148a	94a
T3 (36)	1490a	163a	3b	247a	94a	2b	1371a	39a	30ba
Total oil (mg/kg)				10^3 CFU (g^{-1} dry rhizosphere)					
Z0: 968 control	230a	0b	0b	5b	0b	0c	130bc	0c	0c
Z1: 60 916	70b	60a	6a	42b	190a	4a	59ab	106a	4b
Z2: 76086	105b	84a	0b	800a	173a	0c	351abc	93a	0c
Z3: 92096	109b	94a	7a	370b	141a	1.7ab	780a	10b	14ba
Z4: 119373	138b	63a	2b	21b	159a	2ab	5c	93a	134a

[†] Lh: *Leersia hexandra*, Ep: *Eleocharis palustris*, Ca: *Cyperus articulatus*. Z: zone, [††] Different letters within each column indicate statistical differences (Tukey, p = 0.05, n = 4).

rhizobacteria, for the removal of total weathered oil in tropical regions. The coverage of *L. hexandra*, *E. palustris,* and *C. articulatus*, which were exposed for 36 months to different amounts of weathered oil, showed statistical differences ($p = 0.05$) between treatment means. The fact that *L. hexandra* had the largest coverage suggests a better adaptation to gleyed soils contaminated with weathered oil; this response may be related to the stoloniferous growth (Novelo 2006). This growth promotes higher rates of horizontal elongation and helped *L. hexandra* cover more space compared with the sedges *E. palustris* and *C. articulatus*, which multiply by rhizomes (Davidse *et al.* 1994) and grow radially and in clusters. Although *L. hexandra* covered a larger area, it was affected, during T2 (month 24) and T3 (month 36), by the presence of compact soil aggregates covered with tar-like weathered oil. It was noted that this allochthonous material was hard and obstructed the penetration of roots into the soil, which probably caused a lower horizontal growth of roots, and, above soil, of stolons and shoots. This statement is based on previous studies in both field (Trujillo-Narcía *et al.* 2012, 2014a, b) and in plastic tunnel (Rivera-Cruz and Trujillo-Narcía 2004; Rivera-Cruz *et al.* 2012) which showed lower root elongation, physical damage to the bonnet and fewer plant radish (*Rapahnus sativus*), root of legume (*Leucaena leucocephala* and *Mimosa pigra*), grass (*Echinochloa polystachya* and *Brachiaria mutica*), and sedges (*Cyperus articulatus* and *Cyperus* sp.) caused by penetration resistance of the soil hardness related to the presence of oil, the oil and also for damage to the roots presence of cracks in soil. In a similar study in a mangrove, Ke *et al.* (2002) found that petroleum and oil-coated soil aggregates make them hydrophobic, so that water was not retained by the soil, inhibiting root growth. In addition, in our study the plants *E. palustris* and *C. articulatus* (Table 2) had less coverage and were less adapted to contaminated soils. According to Sarma (2011), these species are dominant in soils that are waterlogged most of the year, and are adapted for survival in microenvironments where limiting factors and high disturbances prevail.

Some native plant species growing in contaminated soils adjacent to oil facilities develop adaptive strategies to withstand stressful conditions, and thus they have the capacity to survive and grow there (Merkl *et al.* 2005; Muratova *et al.* 2008; Maldonado-Chávez *et al.* 2010; Rivera-Cruz 2011a), especially when oil concentrations are low (Lin *et al.* 2002; Olson *et al.* 2007; Liao *et al.* 2015) or high, greater than to 80000 mg/kg of total weathered oil (Rivera-Cruz 2011a; Razmjoo and Adavi 2012). In this research, the grass *L. hexandra* accumulated the largest amount of dry matter compared with the two sedges. However, when compared to the data of the control soil, the old oil inhibited the formation of dry matter in the grass at 12, 24, and 36 months of exposure, unlike *E. palustris* and *C. articulatus*, which showed a positive relationship between dry matter and the amount of oil in the soil. For example, when comparing the data of dry matter of the Month 36 between the control soil (Z0) and the contaminated soils of Z1, Z2, Z3, and Z4, the dry matter decreased 30, 1.5, 25.5, and 21%, respectively (Table 2). Previous studies conducted for 150 days in plastic tunnel (Rivera-Cruz and Trujillo-Narcía 2004) showed that in the grasses *E. polystachya* and *B. mutica*, established in organic clay soil (Gleysol) with 79500 mg/kg of total weathered oil, the dry matter decreased 32.4 and 44.1%, respectively. Muratova *et al.* (2008) evaluated the toxic effect of 10000 mg of diesel in soil for eight weeks and found 63–97% less leaf biomass in several grasses (*Secale cereale*, *Sorghum bicolor*, *Zea mays*, *Lolium perenne*, and *Festuca pratensis*) and legumes (*Medicago sativa* and *Trifolium pratense*). In general, the variations in the production of dry matter in contaminated soils depends on the type of oil, its concentration, the time of exposure, and the type of plant (Adam and Duncan 2002; Rivera-Cruz and Trujillo-Narcía 2004).

The densities of NFB, PSB, and KSB varied in the three aquatic plants studied. The highest bacterial density was found at 36 months in the rhizosphere of the grass *L. hexandra*; no difference was found in *E. palustris*, while in *C. articulatus* the bacterial density decreased (Table 4). This trend, according to Uren (2001), is associated with the specific physical and chemical characteristics of each rhizosphere according to the plant species; the phytoactive compounds exuded by the root and the availability of nutrients are a limiting factor for microbial colonization. Thus, the high density of rhizobacteria in *L. hexandra* can be attributed to the existence of extracellular enzymes derived from plants and of microorganisms that are essential for the initial degradation of substrates mixed with high molecular weight molecules (Badalucco and Kuikman 2001), as is the case of petroleum hydrocarbons. It is also known (Kirk *et al.* 2005; Gaskin and Bentham 2010) that the microbial biomass increases in soils contaminated with hydrocarbons. According to Xiao-Dong *et al.* (2004), rhizobacteria mitigate the toxic effects of contaminants on the plant during cometabolism by exuding the enzyme 1-aminocyclopropane-1-carboxylic acid; monooxygenases and dioxygenases might also be involved (Pothuluri and Cerniglia 1994), hydrolyzing the weathered oil in the presence of oxygen. This research did not prove the presence of enzymes, which must be done by future studies. However, the presence of rhizobacteria has been reported in several rhizospheres associated with oil; thus, Rivera-Cruz (2011a) found populations of 10^3 CFU of *Azospirillum*, *Azotobacter,* and PSB in the grasses *Cynodon plectostachyus*, *Echinochloa polystachya* and *Paspalum virgatum*, which were evaluated in soils with 25000 and 65589 mg/kg of total weathered oil. Miranda-Martínez *et al.* (2007) reported values of 10.9×10^3 CFU of free-living NFB in *E. polystachya* grown in soil with 30 mg/kg of phenanthrene.

The removal of total weathered oil in the four Z of the farm increased over time, but showed a negative relationship with the amount of oil (Figure 3). The lowest removal at Month 36 was 49.1%, recorded in Z4, which was initially contaminated with 119373 mg/kg; the highest removal was 71%, recorded in Z1, which was contaminated with 60916 mg/kg of weathered oil. The degree of removal may be associated with the chemical composition of the oil, in particular with the higher molecular weight fractions (Altgelt and Boduzsynski 1994). It was found that the rhizosphere of *L. hexandra*, which had the largest coverage (Table 3), and that of *C. articulatus*, whose coverage had a positive association with removal of weathered oil. According to the results of Ebastien *et al.* (2006), who evaluated for two years the grasses *F. pratensis* and *L. perenne*, as well as the legume *T. pratense*, the removal of oil was 60%. OMI (2005) and Fernández *et al.* (2006) stated that the time required for removing weathered hydrocarbons (long chain saturated, cyclic, and polycyclic compounds; asphaltenes; and resins) is long because they degrade slowly due to their hydrophobic nature, and low solubility and adsorption to the soil matrix (Chi-Yuan and Krishnamurthy 1995; Huesemann *et al.* 2004; Maletić *et al.* 2011). The importance of the rhizosphere in the degradation of weathered and fresh oil has been previously demonstrated (Schnoor *et al.* 1995; Merkl *et al.* 2005; Basumatary 2012). The rhizosphere is the space where root exudates stimulate the density of microorganisms and increase the degradation of substances (Gleba *et al.* 1999). Because root exudates have similar structural compounds to several petroleum hydrocarbons (Toyama *et al.* 2001), they promote the growth and establishment of plant growth promoting bacteria that fix free-living N, solubilize P and K, and contribute to increased plant resistance to contamination with hydrocarbons. Xiao-Dong *et al.* (2004) reported that the addition of PGPR to creosote-contaminated soil stimulated plant growth.

Conclusion

Each aquatic plant responded differently to 36 months of exposure to different concentrations of weathered oil. The dry matter decreased due to the toxic effect of oil. The formation of biomass by *L. hexandra* was inhibited by the presence of weathered oil, but the biomass of *E. palustris* and *C. articulatus* increased. *L. hexandra* was the most invasive species in gleyed soils of the wetlands contaminated with oil. The density of free-living NFB, PSB, and PKB in the rhizosphere of the three sedges was bio-stimulated by oil; in particular, the rhizosphere of *L. hexandra* showed the largest amount of bacteria under *in situ* conditions at 12, 24, and 36 months. The decontamination of gleyed soils, according to the degradation values, increased with decreasing concentrations of oil, and showed a negative and a positive relationship with the coverage of *L. hexandra* and *C. articulatus*, respectively. It is advisable to carry out phytoremediation tests *in situ* with *L. hexandra*, which showed tolerance to high levels of weathered oil under wetland conditions. Establishing a relationship with physical and chemical factors will allow us to explain the workings of the natural attenuation of weathered oil. The use of tropical grasses in their natural, semi-aquatic environment, given their perennial status, abundant fibrous roots, and adaptations to flood conditions, has potential for the natural attenuation of weathered oil, and also allows the establishment of beneficial rhizobacteria that promote humification and the degradation of hydrocarbons.

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