Chapter 3

Chronic Toxicity of Weathered Oil-Contaminated Soil

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Additional information is available at the end of the chapter

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1. Introduction

Currently, the activities derived from the oil industry, such as extraction, transportation and processing of oil, have affected natural resources [1-3]. For decades, tropical lands have been contaminated by chronic oil spills, causing significant changes in physical and chemical characteristics of the soil, affecting plant development and reducing the growth of microorganisms [4]. Moreover, oil weathering and adaptation of some plants may hide the toxicity of high molecular weight hydrocarbons to other organisms. For this reason, it is necessary to incorporate aspects of *chronic toxicity of weathered oil-contaminated soil* in the study of "*Soil Pollution*". This chapter aims to examine the chronic effects of old spills on soil, plants and beneficial microbes, in order to support the creation of new remediation technologies, focused on face the new challenges of soil contamination.

Oil pollution is a global problem of increasing importance [5], is estimated that every year numerous spills affecting natural resources of Southeast Mexico, in 2011 were contaminated more than 2,063 hectares as a result of 217 oil spills that affected soil and sea; of which 85 were caused by uncontrolled illegal connections, number which increased 204% from 2009-2011 [6]. With respect to the damages to the ground, in 2012 reported an increase of 87% of leaks and spills, its main causes were corrosion damage and failure of materials in pipelines, in that year 30.07 hectares were contaminated in the Southern Region, joined this, there is lag in the care of cases from previous years. This year, the oil industry closed its operations with a total of 163.63 hectares waiting to be remedied, of which, 39.5% are located in the southeast [7].

The environmental problems caused by oil spills, is not limited to visible pollution, because there are chronic effects that silently endanger ecosystems, biodiversity and environmental balance, due to bioaccumulation, leaching and extension of contaminants into groundwater with potential effects on all living organisms [8]. In Mexico, current environmental regulations is the NOM-138-SEMARNAT/SS-2003 which establishes the maximum permissible limits of hydrocarbons in soil and specifications for its characterization and remediation [9], although in this standard are omitted criteria for assessing chronic effects on soil microorganisms and plants as a result of weathered oil contamination. However, the contributions of several studies show that pollution and waste generation caused by oil activities have deteriorated soil quality [10,11]; ie, the social responsibility of the industry and the government should not only attend acute environmental emergencies [12].

Crude oil is a complex mixture of thousands of compounds that when released to the environment, is subjected to physical, chemical and biological processes called weatherability [13]. This process includes adsorption, volatilization, dissolution, biodegradation, photolysis, oxidation, and hydrolysis. The effects of weathering are difficult to predict because it depends on many biotic and abiotic factors [14]. Therefore, the mobility of hydrocarbons is also influenced by natural factors [15], which involve: a) chemical processes (hydrolysis, oxidation, reduction, photolysis), b) transport and physical processes (adsorption, advection, dispersion, diffusion, volatilization and dissolution), and c) biological processes (biodegradation, metabolism and toxicity) [16, 17].

When an oil spill occurs, it covers the soil surface, but initially at high viscosity prevents penetration towards the subsoil. The oil is retained in the topsoil, during this phase, the light fraction is photo-oxidized and volatilized through the soil pore space and transported to the atmosphere, in this process involves the first n-alkanes (methane, ethane, propane and butane), which are evaporated in less than 24 hours in tropical climates [18]. The hydrocarbons which are not evaporated are incorporated into the soil to form a waterproofing layer that prevents the normal flow of water. This layer or film affects the structure, porosity, absorption and water penetration into the soil [19].

Subsequently, the oil soluble fraction diffuses into the soil solution through infiltration. The behavior of this fraction in the soil depends on the type of texture. The presence of fine texture allows the volatilization of some compounds (C5-C7), but when the texture is coarse, it can leach out and transport themselves to the groundwater, affecting other organisms [20], including the human [21-23]. Otherwise, clay soils rich in organic matter immobilize some compounds, reducing their toxicity and decreasing its spread and leaching through the soil profile [24, 25].

The most stable fraction of crude oil is composed with more than 18 carbons (Polycyclic Aromatic Hydrocarbons [PAHs] and polar compounds), which are adhered on the soil matrix decreasing the solubility and volatility [26], and increasing the capacity of adsorption on the mineral and organic fractions, owing to the high content of active surface of the soil (clay 2:1) and to the high molecular weight hydrocarbons [27]. At this stage, the development of bacteria and fungi have influence on mineralization as part of natural attenuation process, but it is not sufficient to remove all hydrocarbons weathered [28].

Recent studies indicate that the major impediments to the biodegradation of hydrocarbons are the physical and chemical properties of the soil, the degree of contamination and the molecular weight of the compounds (C10-C40), but with Enhanced Natural Attenuation (ENA) may be observed a biodegradation of 26.4% [29] up to 60% with enriched amendments, although the n-alkanes are not removed completely [30]. Therefore, it is important to study the toxic potential, because there are reports indicating that can be bioaccumulated PAHs in vegetables such as *C. pepo sp.* [31].

2. Materials and methods

In southeastern Mexico, the land has been affected as a result of extraction; handling and transportation of oil, there are also zones of oil discharges, which are deposited in the open, without any environmental protection measure [32]. The bad condition of the pipelines and the dispersion through surface runoff of rainwater, resulting from weather conditions become more complicated to calculate the chronic effects of soil contamination by hydrocarbons [10]. Accordingly, this study was conducted in three stages, which were analyzed the chemical and physical properties of the soil, the toxic effects of weathered hydrocarbons on the growth and development of seedlings and after 150 and 240 days of exposure, and finally was evaluated the behavior of beneficial soil microorganisms in rhizospheric soil.

2.1. Effect on the physical and chemical properties of the soil

The study was undertaken at the facilities the *Colegio de Postgraduados*, located in Tabasco, Mexico. The soil with weathered oil was collected within 2 km of the Petrochemical "La Venta" (18 ° 04 '54 "N and 94 ° 02' 31" W), Figure 1. The pollution-free soil according to NOM-138-SEMARNAT/SS-2003 was located in the community of Santa Teresa Arroyo Hondo. The objective was to identify the types of soils and their level of similarity.

For both soils were determined the content of organic matter (Walkley and Black), pH (potentiometry), P and K exchangeable (by extraction with 1N ammonium acetate pH 7, quantification by atomic absorption and emission respectively), CEC (extraction 1N ammonium acetate pH 7, quantification by distillation and titration) and texture (Bouyoucos). The analytical methods used were those indicated in NOM-021-2000-RECNAT [33]. Total Petroleum Hydrocarbons (TPH) were determined by the technique reported in the NOM-138-SEMARNAT/SS-2003 [9].

2.2. Effects on plants

Bioassays to determine the phytotoxicity of weathered oil on two species of legumes (*Crotalaria incana* and *Leucaena leucocephala*) were carried out in the facilities of the Laboratory of Soil Microbiology, Colegio de Postgraduados, Tabasco, Mexico. Two states were studied phenological (plant and seedling), in two treatments: a) soil with 150 $mg.kg^{-1}$ TPH (control treatment) and b) soil with 79.457 $mg.kg^{-1}$ TPH weathered (this has been contaminated by over

25 years). Both soils were characterized as Gleysols, with the same pedogenetic origin, as described in the previous section.

Bioassays were established under a Completely Randomized Design (CRD) with three replications and two legumes, these were selected to be species that grow wild in oiled areas, but the former has tolerance, while the second shows sensitivity to high concentrations of crude oil [34]. In each bioassay was used 208 Protocol of the Organization for Economic Cooperation and Development (OECD) modified according to [35], which allows easily identify the symptoms of stress in the plant.

Seedling bioassays: 50 seeds were sown *C. incana* and 25 *L. leucocephala* by repetition, respectively. Glass containers were used (32 x 22 x 5.5 cm). The number of seeds sown per plant species was calculated according to the size of the seed [36], the viability of the species [37] and the area of the container. Seeds were previously scarified to remove impermeable integument, which constitute a barrier for germination [38]. Scarification consisted of immersing the seeds in sulfuric acid for 15 minutes and washed with tap water subsequent to remove all acid residues [39]. Germination tests were performed to determine the initial seed viability, finding viability standard values in both blocks [40]. The test lasted 30 days and the variables evaluated were mortality, height, root length and dry matter accumulation aerial and root.

Plant bioassays: Bioassays were established plants seedlings 30 days from uncontaminated soil. Subsequently, two of these were transplanted into containers, 15 days after a plant was removed from each container. Exposure of plants to pollutant lasted 150 days to *C. incana* and 240 days for *L. leucocephala*, because of its tolerance respective. The physiological variables were evaluated: height, root length, biomass (leaves and stems), root biomass and number of nodes, leaves and fruits. During both assays was provided with water to field capacity and were not supplied nutrients (N, P and K) to avoid interference on the growth of the specimens. The material was weighed on an analytical balance to obtain the values of DM. The multiple comparison of means was performed by Tukey test (a = 0.05). The numerical results were analyzed with SAS software version 9.1, using PROC GLM.

2.3. Effect on soil microorganisms

Quantification of microorganisms was determined by viable count method for serial dilutions [41] for *Rhizobium* extracts in nodules and Free Living Nitrogen Fixing Bacteria (FLNFB) in rhizospheric soil. Culture media were used combined carbon and yeast-mannitol agar [42].

3. Results and discussion

3.1. Effect on the physical and chemical properties of the soil

The soil profile and laboratory results confirmed that both soils are Gleysols, with the same pedogenetic origin [43] (Table 1), but the contamination had abnormalities in their chemical characteristics (Table 2), the which have been reported in other studies undertaken in the South East (Table 3).

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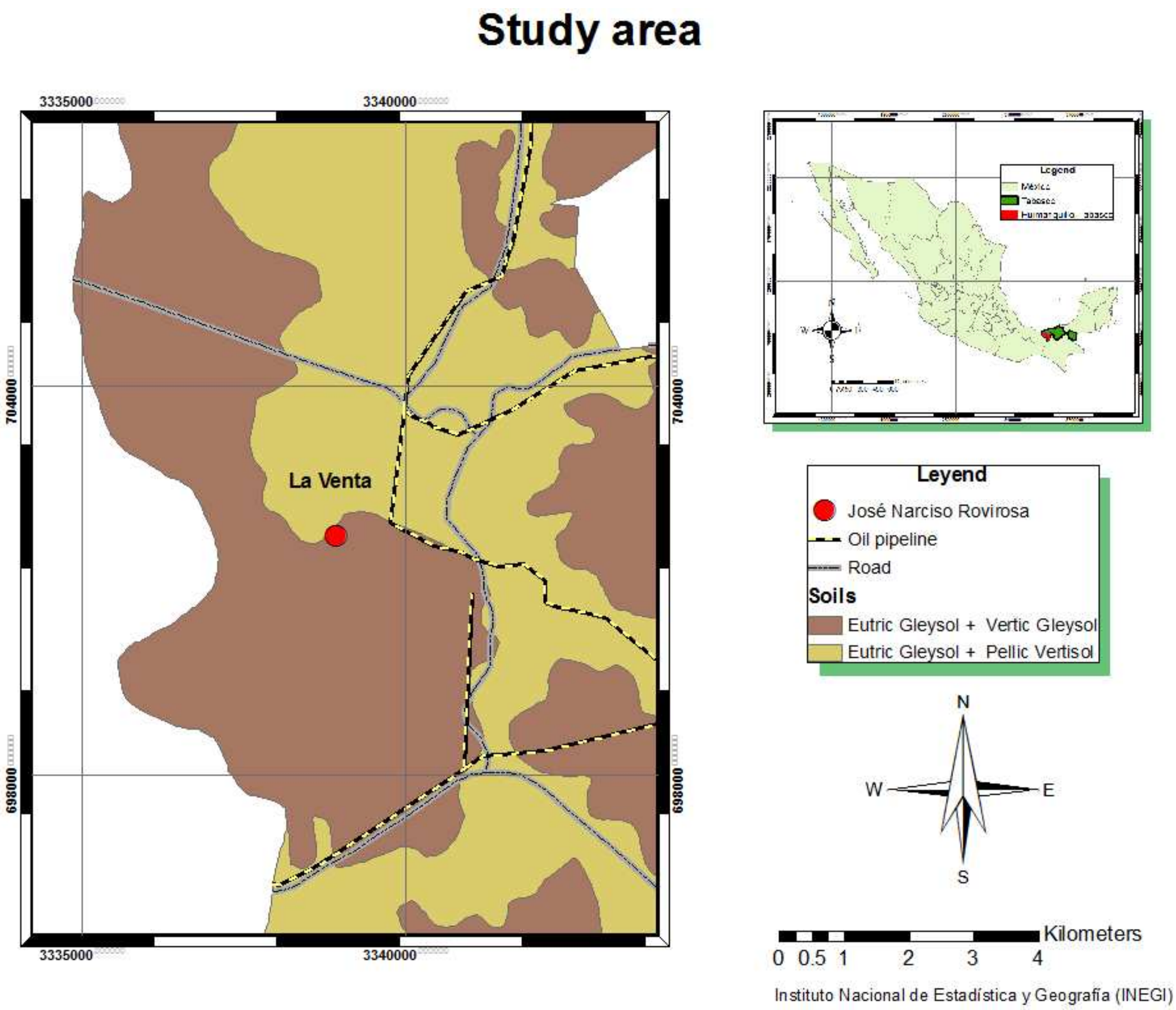

Figure 1. Study zone

Sampling 0-30 cm	pH (H_2O)	OM (%)	P mg.kg^{-1}	K Cmol(+)kg^{-1}	CEC	Clay	Silt (%)	Sand	Textural classification	TPH (mg.kg^{-1})
Soil †	6.3	10.2	23.1	0.35	45.2	61	21	18	Clayey	150
Soil ††	4.2	25.8	3.58	0.4	43.5	48	33	19	Clayey	79,457

* pH 1:2 (potentiometry), organic matter (Walkley and Black), P and K (extraction with 1N ammonium acetate pH 7 and quantification by atomic absorption and emission), CEC (Cation Exchange Capacity) (extraction with 1N ammonium acetate and quantification by distillation and titration), texture (Bouyoucos).

† Control soil

†† Soil with weathered oil (OM without previous removal of TPH)

Table 1. Chemical properties and concentration of TPH in the soils studied.

Sampling 0-30cm	pH	OM	P	K	CEC
		(%)	$mg.kg^{-1}$	C mol(+) kg^{-1}	
Control soil	Moderately acid	Medium	High	Medium	Very High
Polluted soil ††	Strongly acidic	Very high	Low	Medium	Very High

†† Soil with weathered oil (OM without previous removal of TPH)

Table 2. Interpretation of the characteristics of the soils studied. The interpretation was based on the ranges indicated in NOM-021-2000-RECNAT.

Alterations	Effects
High ratios of C / N and C / P	Unfavorable microbial growth [44]
Retaining TPH fractions in organic matter	Alter the solubility of phosphorus [45]
Increasing Na	Limitations in the production of plants [11, 46]
Decreasing pH	Decrease microbionas populations [47]
The electrical conductivity can be increased up to 5.6 times	Salinity [48]
Interruption in the interaction between cations Ca and K	Reduction in capacity of soil to retain Ca and K [32]

Table 3. Alterations reported in soils contaminated with TPH, in southeastern Mexico.

Also, many physical properties of soil are altered, such as water retention capacity; this is because when an oil spill occurs, hydrocarbons bit compete with water filling the pores [49]. On the other hand, increasing the moisture content in the soil reduces the adsorption of liquid hydrocarbons in the organic matter and clay and the filling of the pores and capillaries (Figure 2) [50].

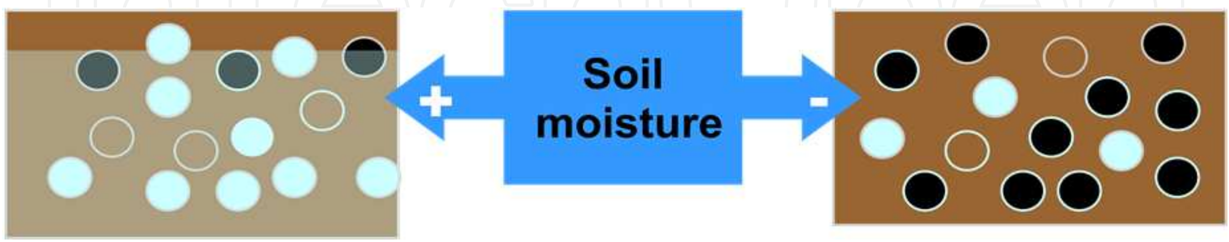

Figure 2. Alteration in the retaining moisture of soil.

The oil can form macroaggregates and macropores that increase water flow (Figure 3), and these changes inhibit the water retention in the soil, which is moistened only after a long period of contact [51], so that the plants may suffer water stress and die.

Figure 3. Formation of macroaggregates in soil with 12,155 mg.kg^{-1} TPH weathered.

3.2. Effects on plants

The results indicated that the lower height and root length were found in soil with 79,457 mg.kg^{-1} of weathered oil, this can be explained because of the limited development of these. Both legumes formed less biomass in soils with higher concentrations of oil, which was associated with lower production of biomass in leaves and stems, as a result of the presence of the contaminant in the soil (Table 4 and 5), which limits the entry of water to the plant. However, the hydrophobic effect also affects the early stages of germination as evidenced with a delay of up to five days in the emergency [52], even in high concentrations of TPH, germination may be inhibited completely [53].

Some authors found that exposure to concentrations of 2 791, 9 025 and 79 457 mg.kg^{-1} of petroleum hydrocarbons in soil inhibited the vegetative growth and reduced plant biomass in seedlings of *Echinochloa polystachya*, *Brachiaria mutica* and *Cyperus sp* [10]. Others establish increased toxicity on the dry weight of rice seedlings (*Oryza sativa*) after 25 days of exposure to 90,000 mg.kg^{-1} weathered oil [54]. Biomass reduction is possibly due to widespread damage, which begins in the root system, hindering vegetative growth and therefore the accumulation of plant biomass. However, the first studies mentions that at low concentrations, oil could stimulate vegetative growth [55].

Crotalaria incana seedlings did not form nodules in soil contaminated with 79,457 mg.kg^{-1} of weathered oil after 30 days of exposure to oil. These results were similar to those obtained according to [34], who found that nodulation was completely inhibited in *Crotalaria sp*. and *Mimosa pigra* by concentrations above 50,000 mg.kg^{-1}.

In both plant species, growth decreased with higher concentration of hydrocarbons in soil (Table 5 and 6). This response may be related to decreased water absorption through the roots for the presence of the hydrophobic film formed by the oil added to the soil [56]. The lack of water absorbed decreases cell turgor, reduces or inhibits the processes of incorporation of nutrients and also affects vegetative growth [57, 58]. Water stress is related to the water potential gradient (which depends on the conditions present in the soil) and the membrane permeability to water (which depends on the species [59] therefore, *Leucaena leucocephala* shown to be more tolerant to water deficit).

Species	Concentration mg.kg^{-1}	Height	Root length	Mortality	Biomass				
					Leaves	Stems	Aerial	Root	Total
		cm		(%)	gr				
Leucaena	150	7.5a	19.0a	0.0a	3.3a	1.3a	4.6a	1.1a	5.7a
	79,457	5.1b	5.1b	4.2a	1.5b	0.5b	2.0b	0.5b	2.5b

Values with different letter are statistically different (Tukey, p = 0.05)

Table 4. Response of *Crotalaria incana* seedlings to 30 days of weathered oil exposure.

Species	Concentration mg.kg^{-1}	Height	Root length	Mortality	Biomass				
					Leaves	Stems	Aerial	Root	Total
		cm		(%)	gr				
Crotalaria	150	9.8a	9.8a	2.6a	1.6a	1.0a	2.6a	0.6a	3.2a
	79,457	2.9b	2.8b	28.6a	1.0a	0.5b	1.5b	0.4b	1.9b

Values with different letter are statistically different (Tukey, p = 0.05)

Table 5. Response of *Leucaena leucocephala* seedlings to 30 days of weathered oil exposure.

Crotaria incana plants showed a greater effect on biomass in response with exposure to the weathered oil (Figure 4), which may be related to increased toxicity of recalcitrants compounds. There are also some soil properties which allow the adsorption of pollutants [60]. Clay soils with high organic content and low pH may favor the persistence of toxic substances in the soil for a long time after the oil spill occurred [61, 34], due to the adhesiveness of organic matter [62].

Furthermore, fine texture allows the oil form a coarse structure on the outside and around the conglomerate making it waterproof [63], this has effects on root development, growth, and as a result will cause decrease in the accumulation biomass [59]. On the other hand, soil contamination by hydrocarbons can also modify some characteristics such as texture, bulk density, ratio of the particle size of the soil, reducing aeration and affecting the productive development of plants [64, 65].

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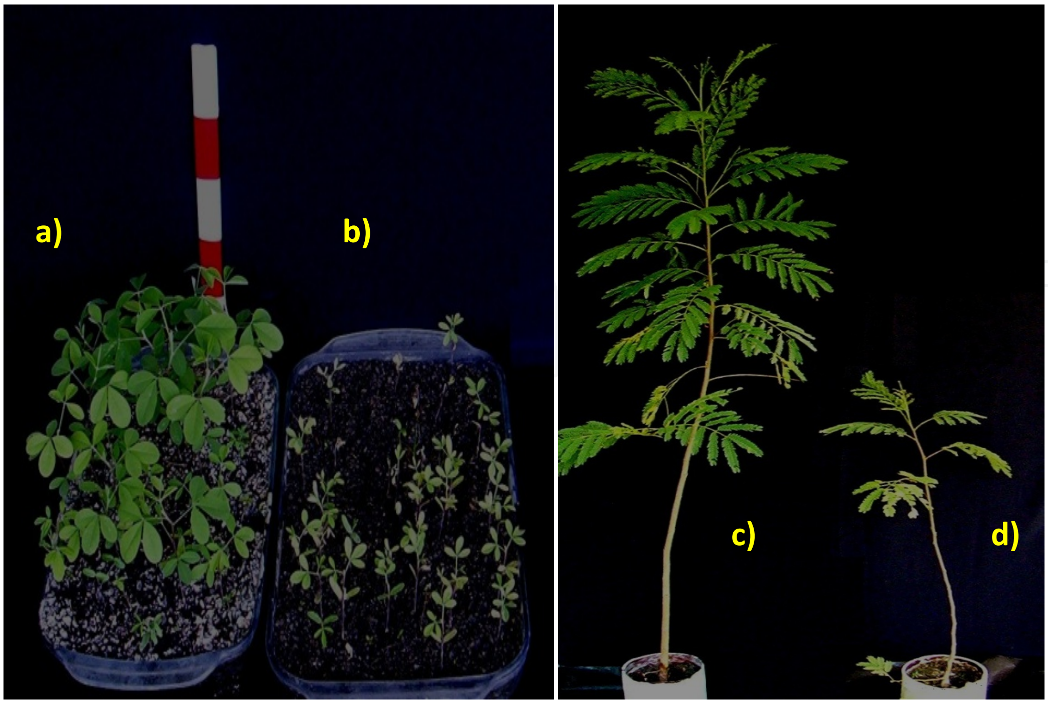

Figure 4. Toxicity on *Leucaena leucocephala* seedlings with: a) 150 $mg.kg^{-1}$ y b) 79,457 $mg.kg^{-1}$ TPH weathered, and plants with c) 150 $mg.kg^{-1}$ y d) 79,457 $mg.kg^{-1}$ TPH weathered.

Species	Concentration	Height	Root length	Knot	Leaves	Fruits	Biomass						
							Aerial	Root	Leaves	Stems	Inflorescences	Seeds	Pods
	$mg.kg^{-1}$	cm		Number			gr						
Crotalaria incana	150	91.7a	17.3a	16.0a	112.7a	54.0a	47.5a	5.6a	11.0a	14.7a	1.5a	9.6a	10.8a
	79,457	32.3b	7.0b	15.0a	2.7b	0.0b	0.6b	0.2b	0.1b	0.6b	0.0b	0.0b	0.0b

Values with different letter are statistically different (Tukey, p = 0.05)

Table 6. Response of *Crotalaria incana* to 150 days of weathered oil exposure.

Species	Concentration	Height	Root length	Knot	Leaves	Fruits	Biomass						
							Aerial	Root	Leaves	Stems	Inflorescences	Seeds	Pods
	$mg.kg^{-1}$	cm		Number			gr						
Leucaena leucocephala	150	149.0a	107.7a	43.7a	19.1a	--	88.6a	34.0a	19.1a	69.5a	--	--	--
	79,457	66.7b	7.3b	31.0b	4.6b	--	15.9b	6.9b	4.6b	11.3b	--	--	--

Values with different letter are statistically different (Tukey, p = 0.05)

Table 7. Response of *Leucaena leucocephala* to 240 days of weathered oil exposure.

3.3. Effect on soil microorganisms

In plants *Leucaena leucocephala* high concentration of weathered oil did not affect populations of *Rhizobium* into nodules, as happened in the case of *C. incana*, in which both populations were significantly lower in a shorter time exposure (Figure 5). This is because the oil alters the physical and chemical characteristics of the soil, causing the blockage of gas exchange with the atmosphere and affecting microbial populations. Furthermore, the weathered oil is adsorbed in the ground, being less accessible and more difficult to degrade by microorganisms [66, 67]. This may bring a direct impact on rhizobia, because they are aerobic bacteria that remain in the soil as saprophytes, until they infect a radical hair.

Some authors mention that soil conditions have a marked effect on rhizobia, because they can impact the survival and the infectivity of root hairs [68, 69]. However, there are many other factors that influence on effectiveness symbiosis such as specificity and virulence of the bacterium *Rhizobium*, nutrimental factors, soil temperature and pH [70], the latter is of utmost importance because has been reported to decrease significantly in contaminated soil [38]. Added to this there are other factors such as the accumulation of heavy metals and salts that affect soil microbiota [11, 4].

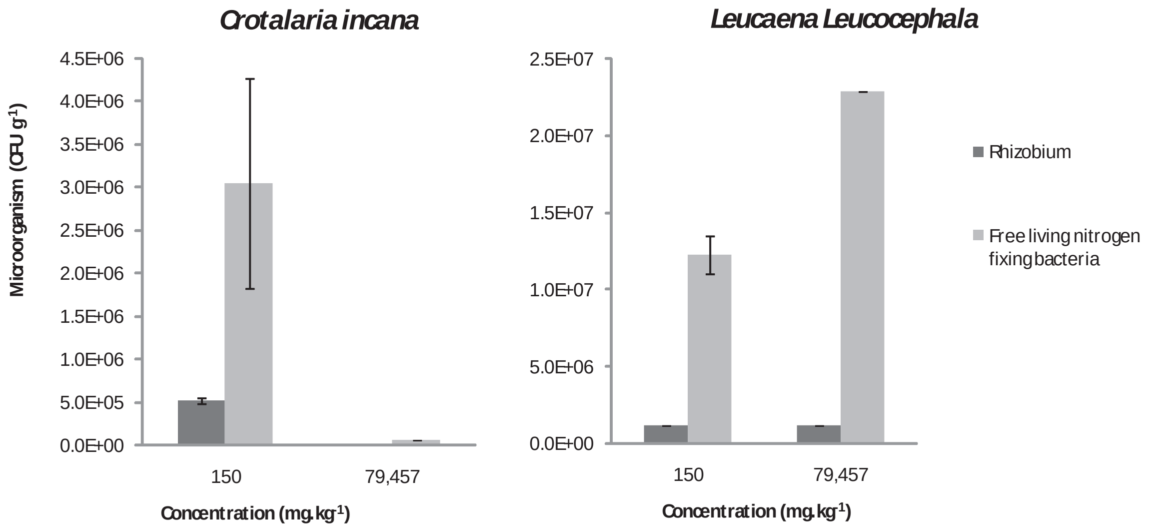

Figure 5. Quantification of populations of *Rhizobium* extracts in nodules and Free Living Nitrogen Fixing Bacteria in rhizospheric soil.

L. leucocephala almost doubled FLNFB populations with 79,457 mg.kg^{-1} of weathered oil. This can be explained because in tolerant species, some microorganisms can increase their populations in the presence of hydrocarbons [71], allowing support microbial growth. As well as [72] argue that tolerant plants are promising tools to accelerate the removal of PAH in long term polluted soils, due to their ability to thrive in a contaminated site, and its success is probably influenced by the relative amount of exudates and other compounds within the root, that stimulate microbial growth [73]. On the other hand, several authors argue that changing the C: N: P, the water content and the water retention capacity of clay soils are crucial to obtain the highest rate of degradation of TPH [74, 75].

4. Conclusion

Soil is one of the most valuable resources that humanity has due to the variety of services offered and which depend for food. Currently, there is tremendous competition for land use, either for use as human settlements, commercial, industrial or produce raw materials (wood) and food, so weathered oil pollution is one of the most important challenges for scientists, because not only it is decontaminated, it comes to restoring the quality and safety of the soil, its properties and microbial component vital for production and proper plant development.

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