Feasibility of a Bioremediation Process Using Biostimulation with Inorganic Nutrient NPK for Hydrocarbon Contaminated Soil in Tunisia

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Abstract

This study focused on the processing time reduction of soils microcosm process, during the biological restore of diesel-contaminated soil under natural conditions. Biodegradation activity of hydrocabonoclastic microflora and biostimulation with inorganic nutrient (NPK) were studied through the determination of optimal conditions which improve bioremediation process. Indeed, after 56 days, about 98% removal rate of total petroleum hydrocarbon (TPH) in soil treated by microcosm's technique were observed relative to approximately 2.22×10^7 CFU/g soil of bacterial number. This performance was achieved with microbial (bacteria) metabolism which accompanies biodegradation of hydrocarbons. Moreover, the hydrocarbon fractions (alkanes and aromatics) were rapidly degraded then the other complex fractions according to GC/MS and FT-IR analysis.

Keywords: Soil; Diesel; Hydrocarbon (TPH); Microcosms; Inorganic nutrient; Bioremediation

Abbreviations:

TPH: Total Petroleum Hydrocarbon; FT-IR: Fourier Transform Infrared Spectroscopy; GC/MS: Gas Chromatography/Mass Spectrometry

Introduction

All the operations in the petroleum industries, such as exploration and production of oil, transportation, refining, refined product handling and oily waste management activities pose serious threats such as human health and environmental toxicity. The hydrocarbon spreads horizontally on the ground water surface and partition into ground water, air space and to the surfaces of soil particles [1]. During this partition, hydrocarbon are degraded in soil mainly by bacteria (0.13-50%) of the total of heterotrophic soil microorganisms, and fungi (6-82%) [2,3].

Microbial community structures in hydrocarbon-contaminated soil are influenced by a number of factors, such as soil type, concentration and bioavailability of the contaminants, nutrient contents, temperature, oxygen content and pH [4,5].

Bioremediation, which is defined as a process that uses microorganisms, plants or enzymes in order to transform contaminants in a less toxic species, is a very attractive method due to its costs, and benefits that result from the pollutant mineralization [6].

Various forms of treatment technologies, employed in the bioremediation of diesel-contaminated soil, are reported in the literature [7-14].

Cunningham and Philp investigated several factors bioaugmentation, biostimulation via inorganic fertilizer and bulking agents that influenced the removal rate of diesel- contaminated soil [8]. Frankenberger et al. [7] reduced the petroleum constituents' concentration at a diesel-contaminated site by injected nutrients (nitrogen and phosphorus) and hydrogen peroxide was added to provide molecular oxygen to the subsurface microflora in degrading the petroleum. More so, Chemlal et al. [10] were restored diesel-contaminated soil by using biological process on biopile system, stimulated with nutrients.

The aim of this study is the evaluation of the processing time reduction of soils microcosms' process, by enriching soil diesel-contaminated with inorganic nutrient during the bioremediation process. Then, we studied the microbial metabolism followed by biodegradation of hydrocarbons through the determination of optimal conditions during the bioremediation process.

Materials and Methods

Study area

Soils samples used in this study were carried out in March, April, and May- 2013 at four hydrocarbon stations, AGIL (S_1), AGIL (S_2), TOTAL (S_3) and OIL-LIBYA (S_4), on Ariana governorate to the far northeastern Tunisia (Figure 1). Those stations are at high risk of pollution, principally by hydrocarbon pollutant result of jet fuel, gas-oil, and Hydrocarbon traces from human activity as well as the presence of natural levels in our sites. Samples were collected using sterile 1000 ml polyethylene bags and transported to the laboratory, before undergoing further analysis the samples were homogenized and kept under refrigeration at 4°C.

The climate of Ariana is arid, characterized by dry and warm summers (from June to August), and cool, wet winters (from December to February). The annual precipitation in the region is 220 mm. The annual predominant wind direction in Ariana is northward. The annual average wind speed is 2.5 m/s, while the maximum monthly average wind speed occurs in April with a magnitude of 2.9 m/s. The annual average temperature of Ariana is around 16-19°C.

Figure 1: Location of sampling sites in far north-eastern part of Tunisia.
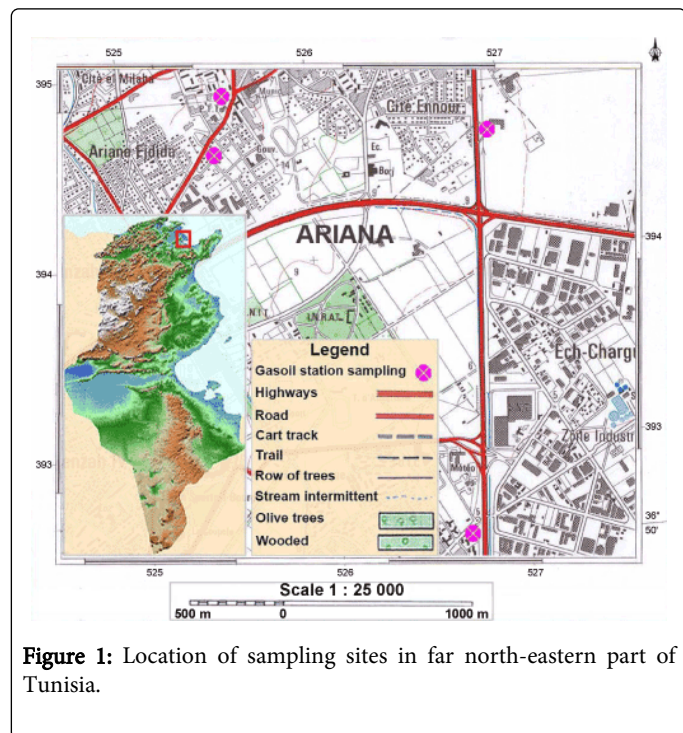
Microcosm's design and study preparation

In order to reduce the soil treatment time using the microcosms, at first, the contaminated soil was enriched by nutrients inorganic NPK with ratio of 10:1:1. The enriched soil was prepared at 1% of the total weight of the contaminated soil, by adding nutrients on the C/N/P ratio of approximately 100/10/1 [15,16].

Sampling soils were divided into three tow-liters sterile flasks each containing 1 kg of soil. The height of the flask was 13 cm with 20 cm internal diameter; every flask has many openings at the base to prevent water logging. Every soil sample occupied approximately half the volume of the flask leaving enough head space.

In the first flasks, the soil sample was thoroughly mixed with a hand trowel sanitized with 80% ethanol. The inorganic nutrient was dissolved in 200 mL sterile distilled water and mixed with the contaminated soil to distribute the nutrient through the soil particles and also to enhance good aeration. In the second flask, the soil was taken without nutrient as control system.

Microcosms were kept at room temperature in a laboratory incubator; nutrient-treated soils were regularly watered weekly with 200 mL sterile distilled water to substitute for evaporated water and also mixed every other day for aeration. The total hydrocarbon compounds were controlled every 7 days, as various physical-chemical and biological analyses.

Analytic analyses

The characterization of the pollution parameters were followed by diverse measurements. Electric conductivity (EC) and pH were measured using a conductivity-meter (Consort C 831) and pH-meter (NeoMet pH 200L) with a glass combination electrode, respectively [17]. Moisture content was determined according to the standard NF M03-002 [18]. The phosphorus (Pi) was extracted in a sodium bicarbonate solution and measured spectrophotometrically [19]. Total nitrogen (Ntot) was measured through the Kjeldahl method [20]. Organic carbon (Corg) was oxidized with a sulphuric acid and potassium bichromate mixture; excess potassium bichromate was titrated with a Mohr salt [21]. The K, Mg, Ca, and Fe contents of the soil were evaluated by EPA methods 7610, 7450, 7140 and 7380 respectively (Soil texture (mass of sand-silt-clay-sized particles) was determined using the ASTM D422-63 method [22-26].

Hydrocarbon analyses

GC/SM analyses

In order to determine hydrocarbons Gaschromatography/massspectrometry (GC/MS) was used. Samples analysis by GC/MS were extracted with Hexane by adding a solvent volume to each sample with 50% of ratio (v/v) and the separation funnel three times, after separation phase, the aqueous phase was removed and kept in a sealed flask for subsequent analysis following US EPA Method 9071B [27].

GC–MS analysis was performed with a HP model 5975B inert MSD, equipped with a capillary DB-5MS column (30m length; 0.25mm i.d; 0.25m film thickness (Agilent Technologies, J&W Scientific Products, U.S.A.). The carrier gas was the helium, and chromatogram peaks were identified by comparing their mass spectra with Wiley and NIST library database and standards of the main components and quantified using the retention time and response factors of these compounds, correlating chromatographic areas to molar concentrations.

FT-IR analyses

The first step, an approximately 2 g of contaminated soil samples were extracted by carbontretrachloride CCl_4 (50 ml). The extraction was performed in a Soxhlet TECH extractor (Soxtec System HT Tecator 1043-Extraction Unit). The final extract was collected in anhydrous sodium sulfate and then filtered in a column containing florisil 60-100 mesh in order to remove polar compounds. About 1.5 mg from the purified extract was compressed under vacuum with 250 mg of KBr. The pellets obtained were dosed by Fourier transform infrared spectroscopy (FT-IR) (SIR MAGNA-IR-560 dualbeam FTIR (Nicolet)) covering a frequency range of 4000-400 cm^{-1}, using CxHx quantification software according to French standard (NFT 90-114) improved by Nicolet company for sediment [28]. This standard allowed the determination of Total Petroleum Hydrocarbon (TPH) extractable by carbon tetrachloride in acidic medium.

Chemicals and media

For microbiology analysis many mediums were prepared, number of total germs was carried out in PCA medium and the Fungi in PDA medium after 7 days of incubation. The minimal medium (MM) used for sub cultivating contained: 0.5 g KH_2PO_4, 0.4 g NH_4Cl, 0.4 g NaCl, 0.33 g $MgCl_2$ ($6H_2O$), and 0.05 g $CaCl_2$ ($6H_2O$) per liter of distilled water, supplemented with 1 ml of trace elements solution [29]. Diesel

was added as a carbon source at 1% (v/v). Solid MM plates presented the same composition supplemented with 18 g/l of Agar.

The pH of the medium was adjusted to 7; all media and solution were prepared with 1 Liter distilled water and autoclaved at 121°C for 20 min. After isolation, and for the working cell banks, the microbial suspension was resuspended in fresh Minimal Medium (MM) containing 15% of glycerol and stored in cryo-vials -80°C.

Results and Discussions

Soil analysis

According to the results of the soil texture analysis the sample soil with medium and relatively permeability rate (Figure 2).
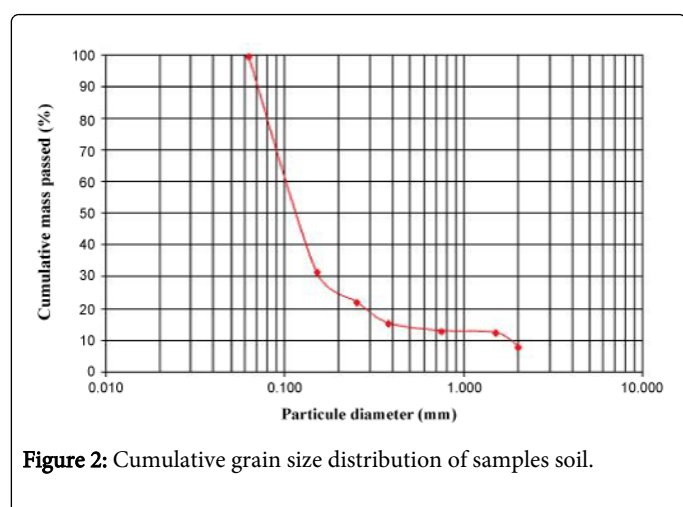

Figure 2: Cumulative grain size distribution of samples soil.

Parameters	Values
pH	6.21
Conductivity (µs/cm)	532
Humidity (wt. %)	14.2
Total nitrogen (g/kg)	2.82
Total phosphorus (g/kg)	1.03
Organic carbon (g/kg)	82.5
TPH (mg/kg)	12700
Number of total germs at 30°C (CFU/g soil)	1.43×10^7
Fungi (CFU/g soil)	2.4×10^6
Hydrocabonoclastic (CFU/g soil)	1.9×10^6
Ca (%)	0.2
Mg (%)	0.3
K (%)	0.2
Fe (%)	2.5

Table 1: Results of physicochemical and microbiological analysis

The guide les of the [30]; suggest that bioremediation is feasible when there is about 10^6 CFU/kg soil of the microbial population. According to the results of the physic-chemical and microbiological analysis were reported in the Table 1. The number of total germs at 30°C (bacteria) is 1.43×10^7 (CFU/g soil), which confirmed that this soil could be treated by biological process using microorganisms.

The GC/MS analysis was performed to identify the presence of hydrocarbon petroleum in the soil sample. The results confirmed that the soil samples consisted mainly of n-alkanes C_{10} through C_{40} approximately lower than 80%, with intermediate branched chain hydrocarbon, along with cycloalkanes, aromatic compounds and other petroleum-based compounds (Figure 3).
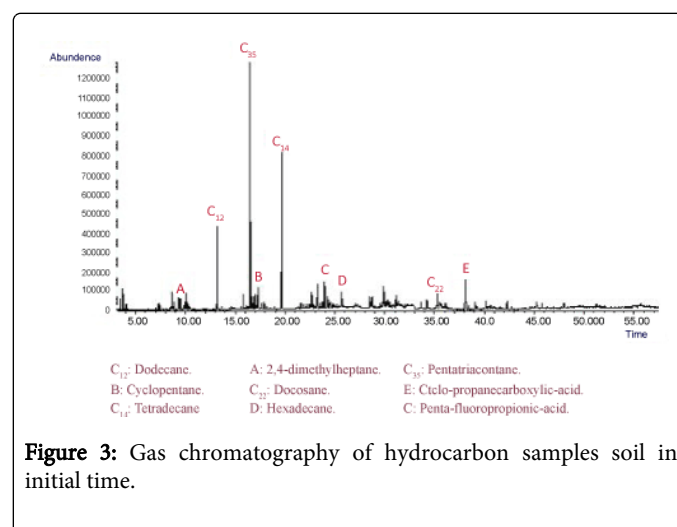

Figure 3: Gas chromatography of hydrocarbon samples soil in initial time.

Figure 4 depicted that the nutrient induced a vigorous microbial propagation which increases after 30 days of treatment from 1.43×10^7 (CFU/g soil) to 2.56×10^7 (CFU/g soil) and from 1.43×10^7 (CFU/g soil) to 1.81×10^7 (CFU/g soil) in the two systems treated and control respectively. The treated system showed decreases progressively during last study, while the control system showed a slowly increase in microbial numbers. This value is considered normal if compared with the results of Wibbe and Blanke [31], who mentioned much higher values.

The Microflora responsible for biodegradation was predominantly bacterial population and this biological microcosms study was done through co-metabolism phenomenon. During the bioremediation process the number of dominant microorganisms increased slightly as the biodegradation ratio.
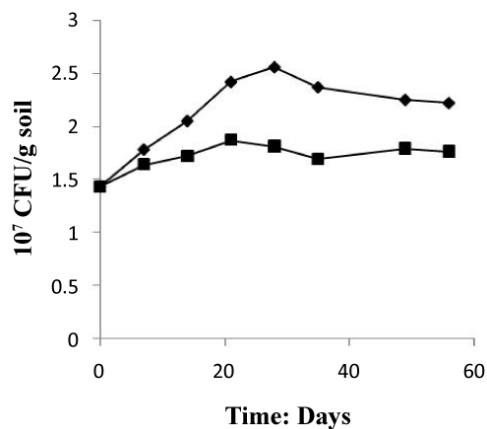
Figure 4: Increase in microbial numbers (CFU/g soil) of the microcosms trial over 56 days (with nutrient, without nutrient).

The pH values were similar within the start of treatment and had an average of 6.2 in two microcosms, treated and control. During the first 10 days of treatment, pH showed an increase from 6.2 to 7.91 and from 6.2 to 6.95 in two systems, respectively. At the end of this period (10 days), the pH ranged from 7.91 to 8.21 and depicted rapid increases in the treated system. At the same, in the control system pH increase from 6.95 to 8.11. After 37 days of treatment, the pH value decreased progressively for the two system and the abatement values reached 91.717% and 97.758%, respectively (Figure 5). The average of pH changing observed was confirmed by same result of Greer et al. [32], that microbial community structures in hydrocarbon-contaminated soil are influenced by a pH value during the biodegradation.
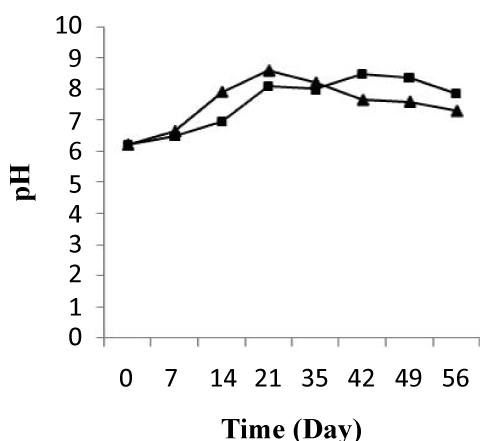

Figure 5: Evolution of soil pH in microcosms during the biodegradation treatment (with nutrient, without nutrient).

In other hand, soil temperature and soil moisture affect the kinetics of hydrocarbon soil reactions during the bioremediation process. Microbial activities in soil involve enzymatic and biochemical processes related to temperature sensitive.

The optimal temperature depends on the volatility and the hydrocarbons solubility pollutants that be treated. In this case, Microcosms were kept at room temperature around 27 ± 4°C in a laboratory incubator in order to facilitate metabolic activity, diffusion, and mass transfer.
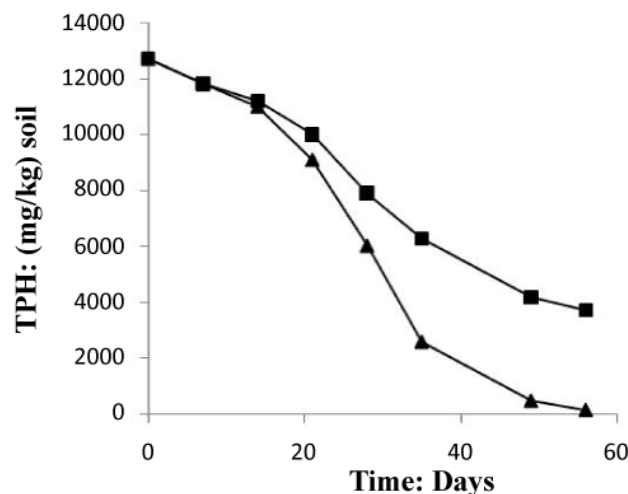

Figure 6: Evolution of Total Petroleum Hydrocarbon (TPH) degradation profile in microcosms experiment during the treatment (with nutrient, without nutrient).

The (Figure 6) mentioned that the TPH concentration decreased slightly from 12700 mg/kg to 11000 mg/kg in the soil treated with inorganic NPK during the first 13 days of treatment. In the control system, the same profile changing was observed where the TPH concentration value ranged from 12700 mg/kg to 11200 mg/kg. In the first system, the TPH concentration decreased rapidly by about 98% at the end of treatment, it is relatively high when compared to the control system which have an average of 67% removal. The removal simulation process is significantly higher if compared to the work of [31] and [10]. Unexpectedly, the differences in hydrocarbon biodegradation between the treated and control system were due to the large differences between bacterial numbers. Moreover, in the other hand The FTIR spectrum (Figure 7) of shows peaks at the frequency level of 3913–3250 cm^{-1} representing –OH stretching of carboxylic groups and also representing stretching of –NH groups. Peak OH groups were observed at 3301.44 cm^{-1}. The peak observed at 2356.35 was indicative of the C–H group. Comparing the final spectrum of the two systems (Figures 8 and 9), it found that aliphatic hydrocarbon C-H bonds are mainly degraded, in contrary the C-H aromatic hydrocarbons bonds (due to bonded OH) are largely degraded during the treatment. This could be explained by the fact that sample aromatic fraction are degraded in the treated system followed by complex fraction. The same result of [33] and [10].

In the most case the removal rate of TPH value obtained depend with macronutrients soil for microorganisms and pollution natural. Soil organic matter is an important source of nutrients for microorganisms, hence decreases in organic matter content with depth are often linked with decreases in microbial population density and decreased ability to degrade hydrocarbon pollution, Moreover, a decrease in organic matter with depth can also reduce the soil's sorption capacity, hence increasing the ability of the pollutant.

Moreover, the aerobic biodegradation of hydrocarbon compound is the modification and decomposition of the compound by soil bacterial to produce ultimately cells, carbon-dioxide CO_2 and water H_2O, this modification is carried out entirely by enzymes located within the microbial cells. This hydrocarbon biodegradation is a biologically catalyzed reduction in complexity of organic hydrocarbon composite through mineralization process. The transformation of composite after its collision with enzymes of the cells depends upon the compound binding to the enzyme and conformational changes at enzyme's active site.
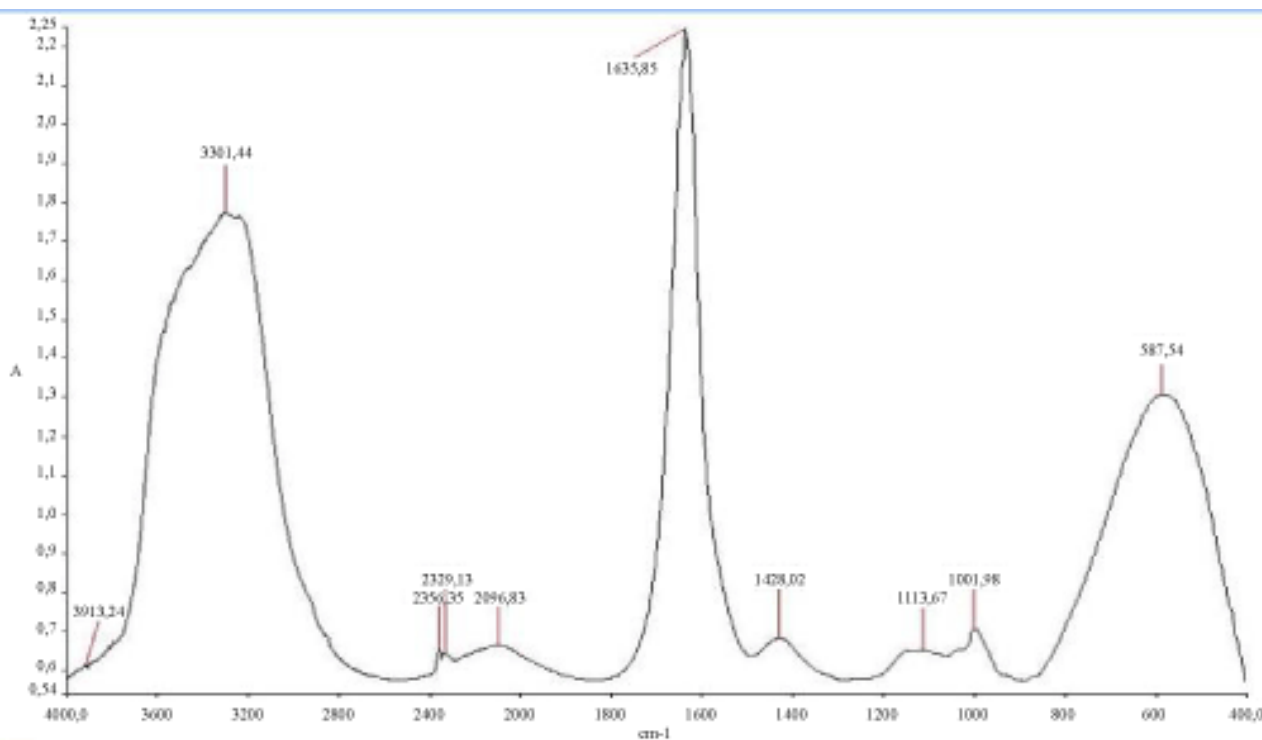

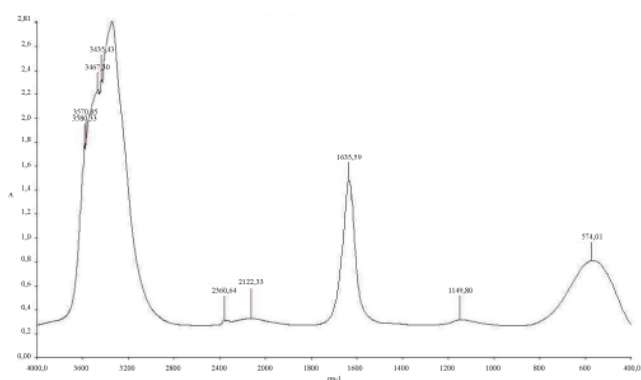**Figure 7:** FT-IR Spectra of total petroleum hydrocarbon for two systems in initial time

Figure 8: FT-IR Spectra of total petroleum hydrocarbon for treated system after 56 days of treatment.
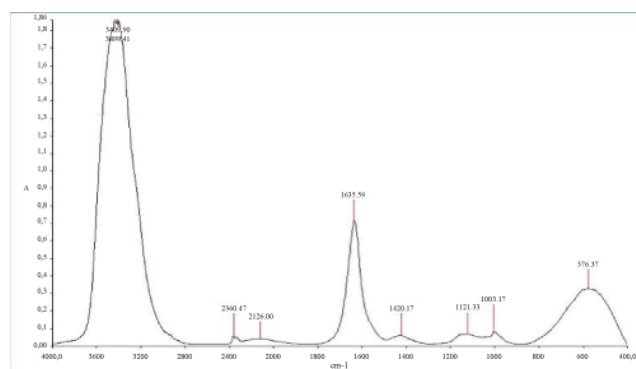

Figure 9: FT-IR Spectra of total petroleum hydrocarbon for control system 56 days of treatment.

Conclusion

This work demonstrated the feasibility of a bioremediation process using biostimulation with inorganic nutrient NPK for hydrocarbon contaminated soil, and underlined the ability of indigenous microorganisms to degrade the diesel through the metabolic activity within the soil. The results of this study confirmed that the

microcosm's process was effective and applicable to Tunisian soil with economic and versatile environmental treatment, which resulted in a major decontamination (about 98%), approximately of 12446 mg/kg soil rate of hydrocarbon removal during 56 days of treatment. However, there is a general need to improve our knowledge about the evaluation of the in situ experimental performances, as factors affecting the ability of the microbes for detoxification and rehabilitation the hydrocarbons in natural environments.

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