Dynamics of CO_2 evolution during bioremediation of clayey and sandy soils contaminated with used lubricating oil

Abstract

The aim of the study was to evaluate the biodegradation of petroleum hydrocarbons in two Brazilian contaminated soil types (clayey S_1 and sandy S_2) at a loading rate of 30,000 and 45,000 mg/kg. A model soil of 300 g with used lubricating oil was amended with commercially available hydrocarbon degrading microbial consortium: Amnite p1300 as the bioaugmentation (T_1), other treatments consist of nutrients amendments - $(NH_4)_2SO_4$ and K_2HPO_4 (NPK) as biostimulation (T_2), unammended soil - natural attenuation as (T_3) and the control soil treated with sodium azide (NaN_3) as (T_4) were evaluated on the microbial community and the degradation of used lubricating oil. Three microcosm replicated flasks per treatment were incubated, and the performance of each treatment was examined by monitoring CO_2 evolution, microbial activity, and oil degradation rate. In Soil 1, T_1 produced the highest values of CO2 of (1600.20 mg/kg) and (1347.60 mg/kg) while the least values were recorded in the control (T_4) with 89.52 and 102 mg/kg in oil contaminated with 3 and 4.5 % respectively. A similar trend was obtained in the bioaugmented treatment soil (S_2) with the highest CO_2 production in T_1. The best percentage oil degradation was also recorded where the utmost CO_2 production was obtained.

Key words: Bioremediation, hydrocarbons, oil-contaminated soil, commercial bacterial; Carbon dioxide respiration, used lubricating oil.

Introduction

Petroleum hydrocarbons are one of the most frequently encountered pollutants in soil habitats due to the increased usage of petroleum products and the seemingly increasing probability of accidents (Samanta *et al*., 2002). Soil contamination by petroleum products is a widespread problem, with many hotspots of pollution arising from individual spills (Whittaker *et al*., 1995). Hydrocarbons are highly toxic to plants and to living microorganisms and invertebrates (Mendoza, 1998; Andreoni *et al*., 2004), and constitute a potential risk to health, which increases as hydrocarbon resistance to degradation increases (Eibes *et al*., 2006). Damage derived from petroleum hydrocarbon contamination will depend on the concentration of the contaminant. Clean-up of these contaminated sites is an important goal, and bioremediation is a low-input and cheap approach to remove hydrocarbons. The cheap, effective and safe method for reducing hydrocarbon pollution could possibly be done through microbial degradation. This technology accelerates the naturally occurring biodegradation under

optimized conditions through adequate oxygen supply and mixing, by adjusting temperature, pH and water content, performing nutrients amendment (biostimulation) or adding a suitable microbial population (bioaugmentation). Biodegradation of complex hydrocarbon usually requires the cooperation of more than a single bacterial species. This is particularly true in pollutants that are made up of many different compounds such as petroleum compounds and complete mineralization to CO_2 and H_2O is desired. One of the greatest advantages on the study of bioremediation in petroleum hydrocarbons contaminated soil treatment is its cost effectiveness, as compared to some physicochemical techniques, which are expensive and need continuous monitoring in order to attain successful results. Bioremediation has emerged as a good technique for environmental treatment regarding organic compounds, such as petroleum hydrocarbons, due to its flexibility and adaptability in different sites (Ryan, 1991).

Natural soils often contain the microorganisms necessary for degrading compounds formed in nature (Alexander, 1994), but bioaugmentation of the populations may enhance the rate of bioremediation. Furthermore, inorganic nutrient supplementation may speed up the process, because the addition of large quantities of oil results in a high C:N ratio that is unfavourable to microbial activity (Choi *et al*., 2002). Activities of microorganism is essential to nutrient cycling in soils, and any effect which pollution has on soil microorganisms will also affect vegetation development, ecosystem functioning and productivity (Bauer *et al*., 1991; Ladd *et al*., 1996). Soil microorganisms are very sensitive to any ecosystem perturbation, since their diversity and activity are rapidly altered by such perturbation (Schloter *et al*., 2003). The measurement of microbiological parameters, such as soil respiration, microbial biomass, provides information on the presence and activity of viable microorganisms as well as on the intensity, kind and duration of the effects of hydrocarbon pollution on soil metabolic activity; such measurements may serve as a good index of the impact of pollution on soil health (Brohon *et al.*, 2001; Eibes *et al.*, 2006). However, results on the effects of hydrocarbon pollution on microbial biomass and activity are not always coincident, probably due to the differences in chemical properties of the hydrocarbon used (Xu and Johnson, 1995).

A standard test in the initial assessment of biodegradation of any contaminant is the measurement of evolved CO_2 (Sharabi and Bartha, 1993). Carbon (iv) oxide measurements are simple, non-destructive, and representative of ultimate biodegradation. Most studies of the biodegradation of organic contaminants have used CO_2 evolution as a measure of either the

microbial activity or of mineralization (Venosa *et al*., 1992; Huesemann and Moore, 1993). The evolved CO_2, however, may not be derived only from the compound under evaluation. The degradation of indigenous soil organic matter upon addition of any easily degradable organic compound (a priming effect) may also contribute to the evolution of CO_2 (Alexander, 1994). It is also true that contaminants may well serve as organic carbon sources, and an enrichment of oil-degrading microbial populations has been observed in most contaminated ecosystems (Margesin *et al.*, 2000). A better understanding of the effect of hydrocarbon contaminants on plant and soil microorganisms may be of help in assessing the recovery potential of a soil. Therefore, proper control treatments should be included to control such sources of CO_2 evolution. This study aimed to determine an optimal bioremediation strategy by evaluating the effects of addition of oil-degrading commercial bacterial consortium, the application of nutrients, and to fully understand the best strategy for oil-contaminated clay and sandy soils; by measuring CO_2 evolution as an index of mineralization of different contaminated with used lubricating oil hydrocarbons. This study also attempted to determine the relationships between CO_2 evolution and microbial activity or used lubricating oil biodegradation, to confirm the utility of these indices in the evaluation of bioremediation monitoring techniques.

2. Materials and methods

2.1. Soil sample and analysis

Soil surface samples (0–15 cm) taken from two differently textured soils (a sandy and a clayey soil) with different levels of organic matter were collected from two locations in Minas Gerias, Brazil with no known history of petroleum product contamination, A single large core was collected for each soil type from the A horizon, not including the surface litter layer, kept in sack and transported to the laboratory for analysis. The soil was sieved using a 5 mm diameter stainless sieve. The characteristics that were determined using standard techniques are as listed in Table 1. To establish its physical and chemical characteristics, the soil was homogenized (gentle blending) and characterized before treatments application. Used lubricating oil was collected from a gasoline and car service station in close proximity to the University. Amnite p1300 special bacterial strains specially made to degrade used lubricating oil were obtained from Cleveland Biotech Ltd., UK. Nitrogen content of the soil was determined using

Kjeldahl method, the available phosphorus was determined by colometry after Mehlich 1 extraction and organic carbon content was determined by the procedure of Walkley and Black using the dichromate wet oxidation method (Nelson and Sommer, 1992). The pH of the soils was determined in each sampling dates by adding 10 g of soil to 25 ml of distilled water i.e. 1:2.5 (w/v) soil/distilled water in a beaker, stirred with a glass rod, and allowed to stand for 30 minutes. The soil suspension was stirred gently and repeated three times for the determination of the pH using pH meter.

2.2. Experimental design

The artificially contaminated model soils of 300 g were manually mixed with used lubricating oil at room temperature of (25 ± 1 ^{0}C) under laboratory conditions. The soil was spread evenly and thinly in a large glass dish; the oil was added at a level of 30,000 mg/kg and 45,000 mg/kg dry weight of soil (3 % and 4.5 % w/w) respectively, poured evenly over the surface, and then mixed with a stainless steel spatula for 5 min before transferring to the microcosm (one litre (1L) glass flasks sealed with teflon-lined rubber stoppers). Since it is common for authentically-contaminated soils to have similar or higher oil concentrations (Saterbak *et al.,* 1999), the concentrations of the added oil was similar to earlier microcosm studies (Wünsche *et al.,* 1995). The microcosms were used to simulate the comparative effect of used lubricating oil addition and bioremediation using a commercially available hydrocarbon degrading microbial consortium (Amnite p1300), a special bacteria strains consisting a mixture of *Bacillus subtilis, Bacillus megaterium, Pseudomonas putida, Pseudomonas fluorescens, Phanerochaete chrysosporium, Rhodococcus rhodocrous* on a cereal (bran) as the bioaugmentation treatment. The bacteria were conditioned to degrade heavy hydrocarbons. The concentration of biomass in Amnite p1300 was approximately 5 x 10^8 cfu/g of bran. In addition to bioaugmentation using microbial consortium, the polluted soils were amended with ($(NH_4)_2SO_4$ and K_2HPO_4) as biostimulation. The ratios of carbon:nitrogen:phosphorus of the nutrient compound was carefully adjusted to 100:7.5:1 (optimum conditions), similar conditions provided in the biostimulation treatment were adopted in the bioaugmentation treatments plus the addition of Amnite p1300. The unammended soil without addition of nutrients and bacterial inoculums (natural attenuation), was included to indicate hydrocarbon degradation capability of microorganisms naturally present in the contaminated soils (i.e. the autochthonous microbes). There was a control soil in which most of the indigenous bacteria were killed by the addition of biocide sodium azide

(NaN_3) (0.3% w/w) to inhibit soil microorganisms and to monitor abiotic hydrocarbon losses on the microbial community in two different soil types. Microcosms were arranged in a random order, and rearranged every 2 ± 2 weeks throughout the duration of the experiment. Triplicates sample treatments were set up, the content of each container was tilled carefully every week for aeration to take place, addition of sterile distilled water was added every week throughout the 90 – day period to maintan moisture content at 70 % water holding capacity (Pramer and Bartha, 1972).

2.3. Soil respiration

Amended-moist soil with water holding capacity (WHC) of approximately 70 % was incubated in triplicate at (25 ± 1 ^{0}C) under laboratory conditions, in one Litre (1L) glass flasks sealed with teflon-lined rubber stoppers. Measurement of carbon iv oxide evolution was performed through indirect method (Weytjens *et al.*, 1994). Microbial activity was monitored by analyzing CO_2 accumulation in the headspace by gas chromatography. Headspace samples of 1 cm^3 were taken from microcosms with a Hamilton gastight® syringe and were injected into a Gas Chromatograph Model GC- 14B (Shimadzu Crop Kyoto Japan), with a thermal conductivity detector (TCD) at temperature of 150 ° C, injector at 100 º C and Porapak -Q column at 50 º C, using nitrogen as a carrier gas. Respiration rate was expressed as evolved CO_2 in mL CO_2 m^{-2} h^{-1}, and the accumulated CO_2 concentration in mg CO_2/ kg of dried soil. The amount of CO_2 evolved during the mineralization of used lubricating oil was monitored using the accumulated concentration of CO_2 recorded from the CG-TCD. Soil samples were withdrawn from each treatment for the residual analysis of Total Petroleum Hydrocarbons (TPH) and Polycyclic Aromatic Hydrocarbons (PAHs) at every 15 days intervals to the end of the experiment. Microcosms were aerated for 15 min after CO_2 measurement to maintain oxygen levels in the system. Microcosms were set up in triplicate in tightly closed glass flasks. To determine metabolic activity in each microcosm, respiration through CO_2 emission monitoring were periodically performed. The respiration mean of the blanks was subtracted from the treated microcosms, and the difference in CO_2 production between the blanks and the treated microcosms was used as the amount of CO_2 produced.

2.4. Enumeration of bacteria

The study was conducted at room temperature and the enumeration was carried out at 0, 15, 30, 45, 60, 75 and 90 days. To monitor cell numbers and biodegradation, 1 g of soil was removed from each microcosm at the set times and suspended in 9 mL of saline solution in sterile centrifuge tubes. The mixture was vigorously shaken on a vortex mixer for 3 minutes and then the soil particulates were allowed to settle for 1 min before 0.1 mL of the supernatant fluids were sampled for CFU counts. The number of colony-forming hydrocarbon-degrading bacteria (HDB) was attempted by plating three replicate samples from each treatment withdrawn every 15 days on a mineral medium containing used motor oil as the sole carbon source. The mineral medium contained 1.8 g K_2HPO_4, 4.0 g NH_4Cl, 0.2 g $MgSO_4.7H_2O$, 1.2 g KH_2PO_4, 0.01 g $FeSO_4.7H_2O$, 0.1 g NaCl, 20 g agar, one percent (1%) used engine oil in 1,000 mL distilled water, and the medium was adjusted to pH 7.4 (Zajic and Supplission, 1972). The oil agar plates were incubated at 30°C for 7 days before the colonies were counted.

2.5. Statistical data analysis

Data collected were subjected to statistical analysis using general linear model of analysis of variance (ANOVA). Significant treatment means were compared using Tukey test at $P > 0.05$ (Statistical Software 8.0: Stat. Soft, 2007).

3.0 Results and Discussion

Table 1 shows the physico-chemical properties of the non-contaminated soil sample used for the experiment.

Table 1: Selected physical and chemical characteristics of the noncontaminated soil samples

Parameters	Soil 1 (S_1)	Soil 2 (S_2)
pH (H_20)	5.20	4.92
Total Nitrogen (%)	0.43	0.11
Avail. P (mg/dm^3)	1.00	0.40
Organic C (%)	3.50	0.81
C:N ratio	8.14	7.56
Moisture Content (%)	33.80	11.30
Sand (dag/kg)	11.00	68.00

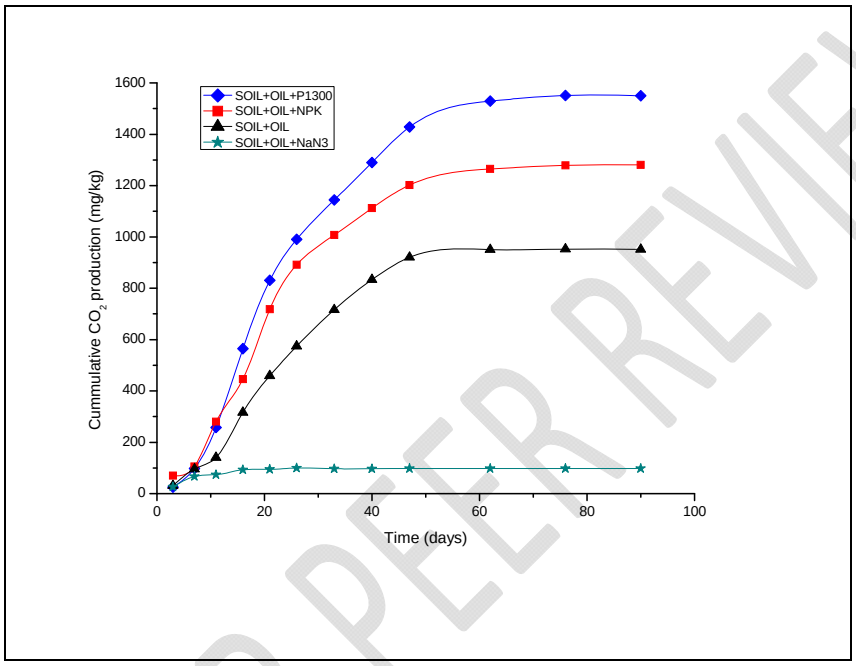
Silt (dag/kg)	9.00	4.00
Clay (dag/kg)	80.00	28.00
Texture	Clayey	Clay loamy sand
Soil Type	Red latosol	Red yellowish latosol

3.1 Soil respiration (CO_2-evolution) analysis. The values of CO_2 evolved during a 90-day incubation experiment from soil samples at different times in 3.0 % and 4.5 % level of contamination are shown in Fig. 1 and 2 respectively. Dynamics of CO_2 emissions were higher in the clayey soil than in the sandy soil both for the contaminated and the control treatments. This occurrence might be as a result of the higher nutrient contents and microbial metabolism in this clayey soil which has been shown to offer greater capacity for physicochemical attenuation of contaminants than coarse sands. (Pye and Patrick, 1983).

The contaminants at the two loading rates (3.0 and 4.5 %) assayed, increased soil respiration especially in the clayey soil. The used lubricating oil in clayey contaminated soils showed that respiration in 3.0 % loading rates producing the greatest CO_2 emissions of (1600.20 mg/kg) and (1347.60 mg/kg) while the least values were recorded in the control (T_4) with 89.52 and 102 mg/kg in soil contaminated with 3 and 4.5 % respectively. The same noticeable trend was recorded in S2 where T1 produced the highest CO2 production of 932 and 702 and the T4 recorded the least values of 113 and 64 mg/kg for 3 and 4.5 % respectively. The high amount of CO_2 liberated in clayey soil amended T_1 is an indication of high utilization of organic carbon nutrients from the petroleum hydrocarbons present in this sample. The exception to this trends was that respiration rate of the samples with higher contamination level of 4.5 % used lubricating oil was lower compared with the soils contaminated with 3 %. The stimulatory effect of used lubricating oil on soil respiration persisted in both soils throughout the incubation period, and was still noticeable 90 days after contamination (Fig. 1 and 2). The cumulative evolution of CO_2 in the clayey soil (S1) at both level of contamination (3.0 and 4.5 %) in the amended treatments with Amnite p1300 products in (T1) were higher than treatments amended with nutrients (T2) in (3.0 and 4.5 %) level of contamination. The lower values recorded in both soils contaminated with 4.5 % might be due to the toxicity of the used lubricating oil to the microorganisms present in the contaminated soils. Soil respiration (in terms of carbon dioxide - CO_2-evolution) in T1 and T2 were significantly higher than in T3 and T4. The CO_2-evolution in T4 reached maximum

values after 30 days, decreasing thereafter and remaining almost constant till the end of the 90-day experiment. Carbon dioxide (CO_2) evolution in T4 indicates that the sodium azide (biocide) used for this experiment was not 100 % effective to inhibit the carbon oxidising bacterial metabolism during the experimental period.

S1



S2

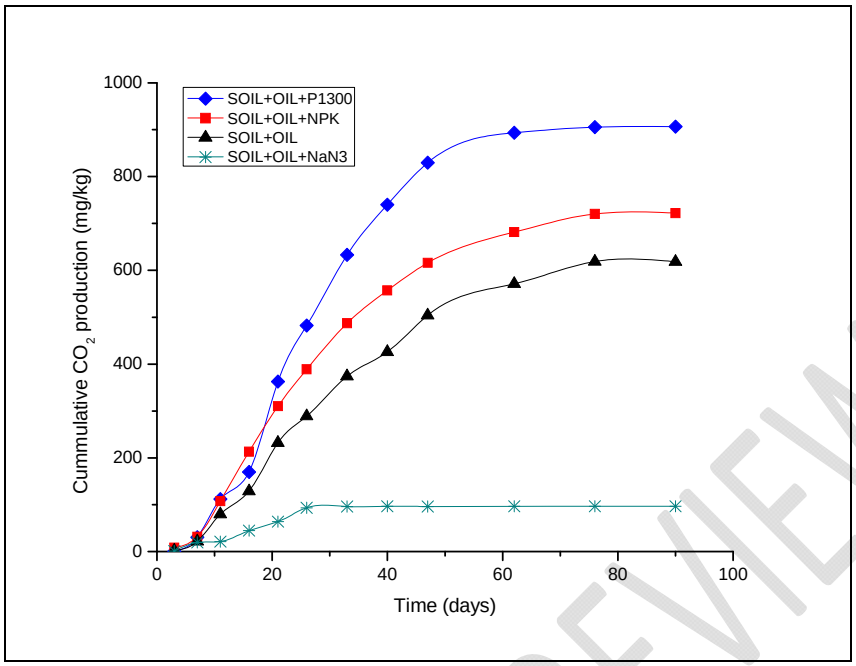
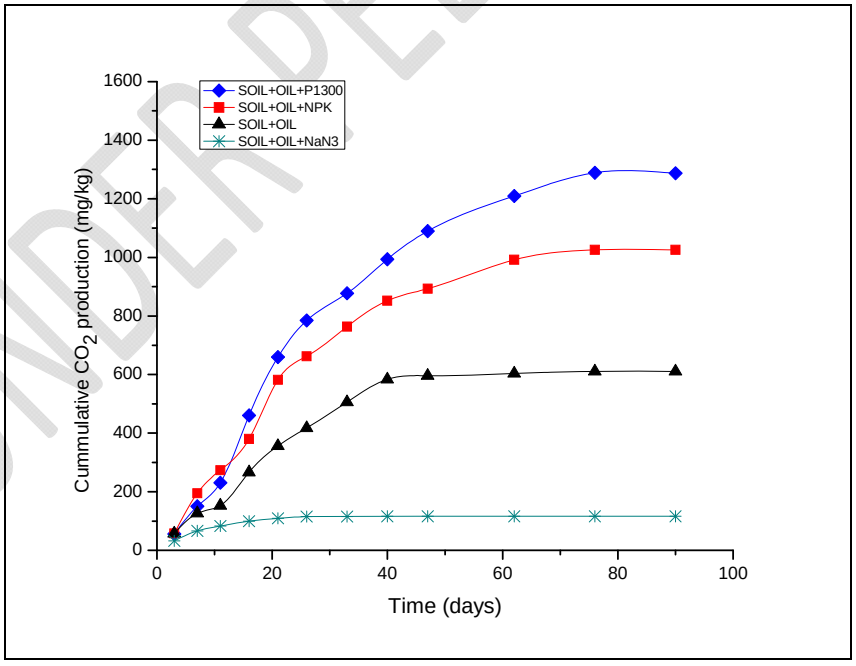


Fig. 1: Cumulative CO_2 production during biodegradation of clayey (S1) and sandy (S2) soils contaminated with 3 % used lubricating oil.

S1



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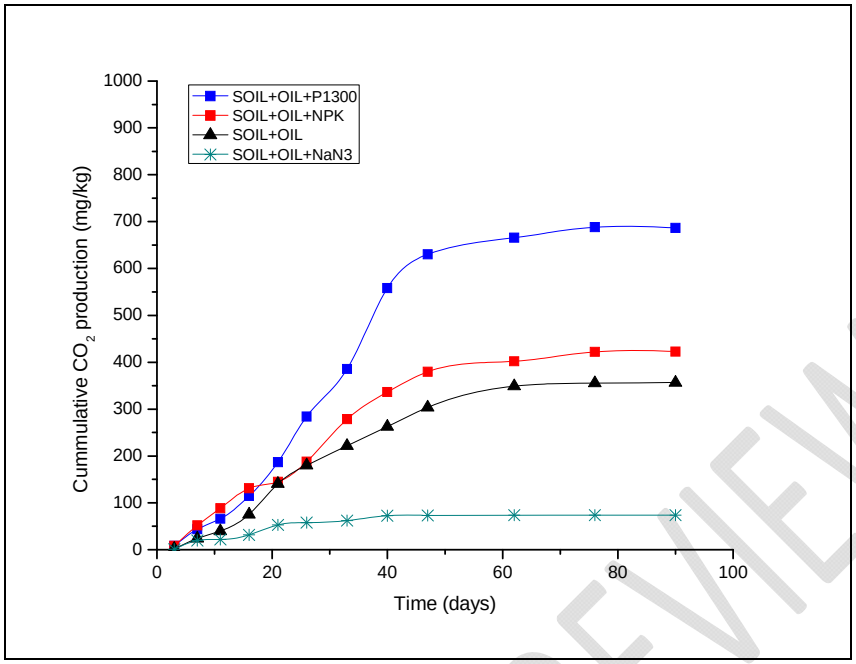
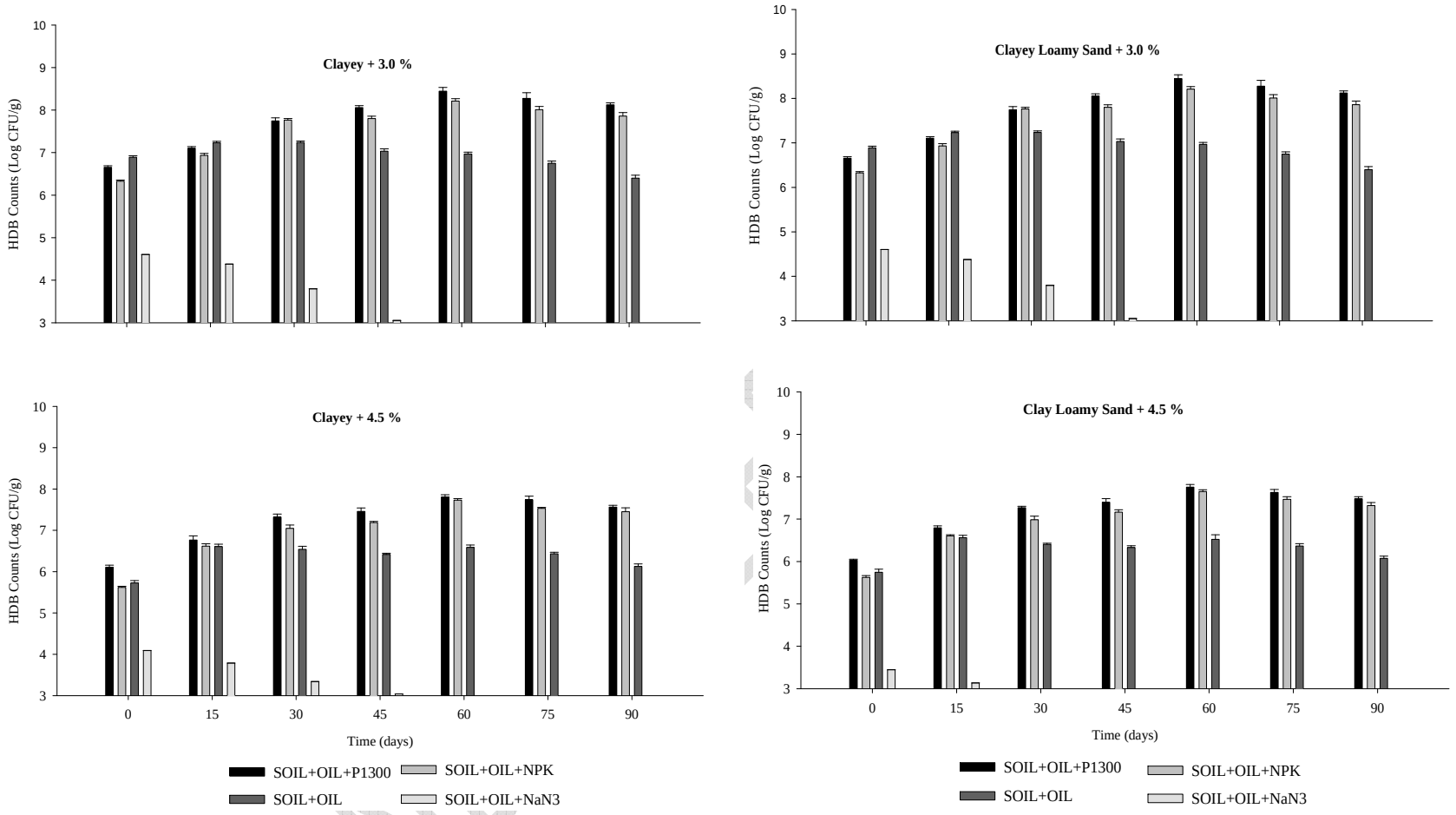


Fig. 2: Cumulative CO_2 production during biodegradation of clayey (S1) and sandy (S2) soils contaminated with 4.5 % used lubricating oil.

3.3. Enumeration of hydrocarbon degrading bacteria. The hydrocarbon degrading bacterial (HDB) counts in T1 contaminated with 3 % ranged from (4.5×10^6 to 2.8×10^8 CFU/g) in S1 and (4×10^6 to 2.5×10^8 CFU/g) in S2. The hydrocarbon degrading bacterial counts was slightly higher in S1 than S2. A similar trend was recorded in soil contaminated with 4.5 % in T1, with lower microbial population ranging from (1.28×10^6 to 6.4×10^7 CFU/g) in S1 and (1.08×10^6 to 5.7×10^7 CFU/g) in S2. The reason might be due to the ability of the clay properties to surface adsorption and microbial metabolism of active organisms present in clay than in sandy soils. (Pye and Patrick, 1983). The low percentage of contamination with (3.0 %) used lubricating oil might be the reason for the relatively high and progressive biodegradation in the soil. The low percentage oil contamination appeared not to pose serious challenge to the metabolic activities of soil microorganisms. The population of hydrocarbons degrading microbial counts were highest in T1 followed by T2 and T3. Control T4 has the least counts in both soils used for the experiment. This result clearly demonstrates the benefit of bioaugmentation of oil polluted soil with aminte p1300 products. An enhanced comparison between the four treatments investigated in this work is revealed in fig. 1. In the control treatment (T4), most of the

indigenous bacteria were killed with a biocide (NaN_3). The number of hydrocarbon-degrading microorganisms increased with time both at the contamination levels and the two soils. Already after 15 days, the counts of degrading bacterial consortium on used lubricating oil hydrocarbon showed that soil microorganisms adapted rapidly to the hydrocarbon contamination and were able to utilize the used lubricating oil as carbon source (Fig. 3). The counts of hydrocarbon degrading bacteria (HDB) in both level of contamination (3.0 % and 4.5 %) in T2 were lower than T1, but, appreciably higher compared to T3 and T4. The reason for higher counts of bacteria in T2 soil might be as a result of presence of appreciable quantities of available nutrients added, which are necessary for bacterial biodegradative activities (Abdulsalam *et al*., 2011).



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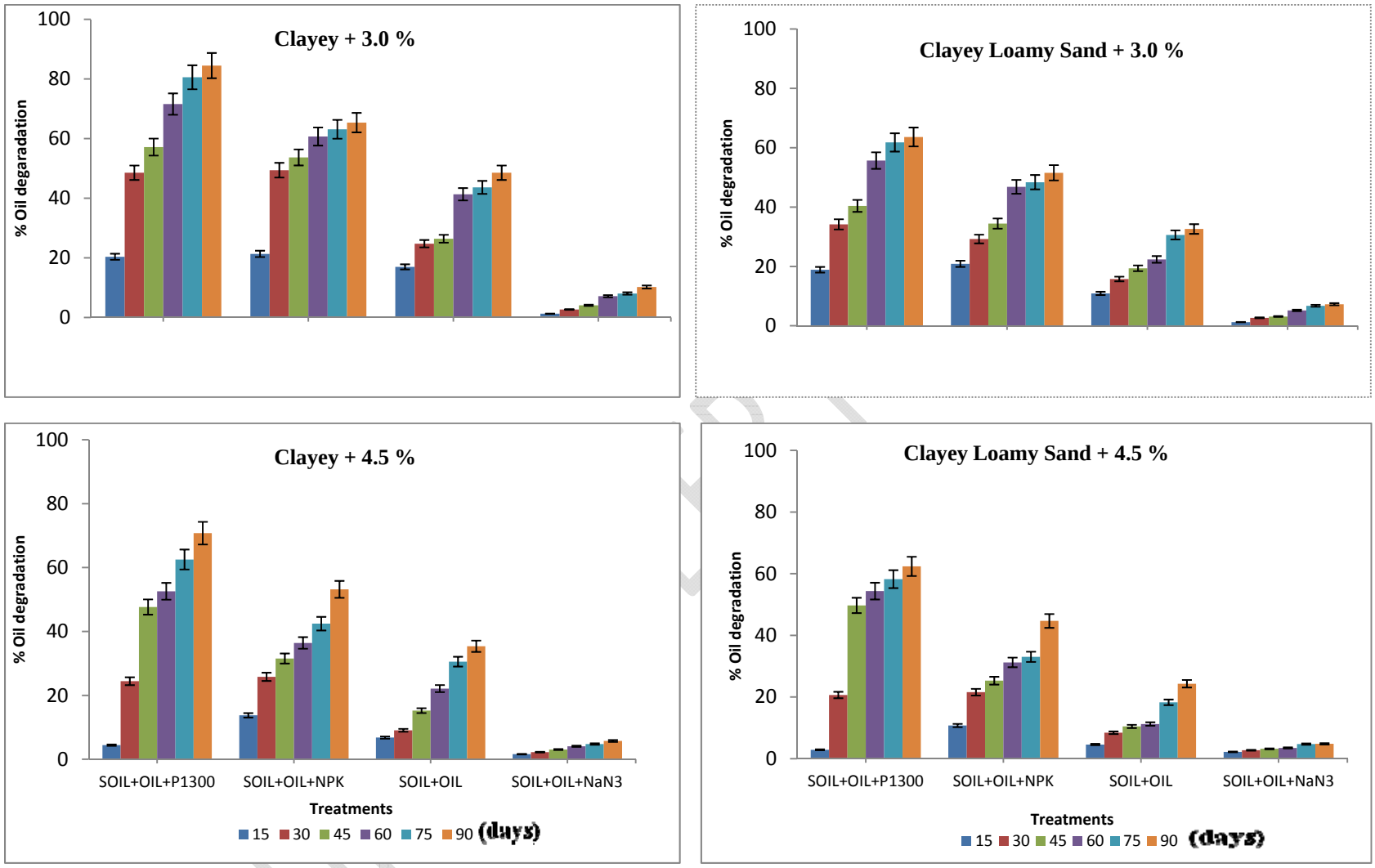
282 Fig. 3: Hydrocarbon-degrading bacteria (HDB) counts in soils contaminated with 3.0 and 4.5 % used lubricating oil. Vertical bars
283 indicate standard error of the means SE (n = 3)

3.3. Biodegradation of used lubricating oil. Following a better biodegradation extent in our earlier studies using only one contamination level in three types of soils (Adeyemo *et al*., 2012), the biodegradation of used lubricating oil was studied using Aminte p1300, a commercial bacterial consortium, was investigated. Trends in the biodegradation percentage in the soil contaminated with 3.0 % and 4.5 % used lubricating oil are shown in Fig. 4 The results showed the highest biodegradation percentage of (84 and 64 %) in soil 1 (S_1) and soil 2 (S_2), respectively, contaminated with 3.0 % of the used lubricating oil amended with Amnite p1300 (T_1) compared to (65 and 52 %) in T_2; (49 and 33 %) in T_3 and (10 and 7 %) in T_4 at the end of 90-day experiment. A higher degradation percentage observed in S_1 compared to S_2 in this study might be due to the higher clay contents of S_1. Soils of high clay contents have a greater affinity to adsorb more nutrients than the coarse sandy soils (Pye and Patrick, 1983). The low percentage of contamination with (3.0 %) used lubricating oil might be the reason for the relatively high and progressive biodegradation in the soil, because it does not pose a serious challenge to the metabolic activities of soil microorganisms. It may also be due to the mixed bacterial consortium that is present in T_1 that combine individual's effect of the bacterial strains for better oil degradation (Rahman *et al.,* 2002; Ghazali *et al.,* 2004). With the increase in the concentration of the contaminated soils (4.5 %), lower percentage of degradation (71 % and 62 %) was observed in S_1 and S_2, respectively, in T_1 compared to the previous level of contamination (3.0 %). Similar trends were recorded in soil contaminated with 4.5 % in T_2, T_3 and T_4 (53 and 45 %), (35 and 24 %) and (6 and 5 %) respectively. This may be attributed to the toxicity of the oil on the microbial flora of the soil and thus the high concentration of oil which might likely had negative effects on the biodegradative activities of the microbial population in the contaminated soil. (Adesodun and Mbagwu, 2008). Higher degradation was also observed in our previous work (Adeyemo *et al.,* 2012) with reduced concentration of the used lubricating oil in soil following application of microbial consortium (Amnite p1300). The result is in agreement with the findings of Rahman *et al.*, (2002) who reported decrease in the rate of biodegradation of crude oil, as the concentration of oil increases. Some removal of hydrocarbons was also seen in the soil which was not amended with commercial bacterial consortium. This removal could be attributed to the combined actions of indigenous microbial population stimulated by the

addition of nutrients to the polluted soil as well as abiotic weathering such as evaporation, photochemical oxidation, and adsorption onto particulate material.



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319 Figure 4: Percentage degradation of petroleum hydrocarbon in soil contaminated with 3.5 and 4.5% used lubricating oil in soil 1 (S1) and Soil 2
320 (S2). Vertical bars indicate standard error of the mean SE (n = 3).

4. 0 CONCLUSIONS

Hydrocarbons degrading bacteria (HDB) counts were higher in clayey soil, at highest population of (2.8×10^8 CFU/g) at day 60, in 3.0 % contamination level, though the population reduces as the contamination level increases. This study has also shown that soil microbiological parameters may be useful tools for assessing the effect of hydrocarbon contamination on soil wellbeing. The contaminants at the two loading rates (3 and 4.5%) assayed, increased soil respiration in both soils, especially in the clayey soil. The used lubricating oil contaminated clayey soils showed that respiration in 3.0 % loading rates producing the greatest CO_2 emissions. The higher amount of CO_2 liberated in clayey soil amended with amnite products and contaminated with 3 % and 4.5 % used lubricating oil is an indication of high utilization of organic carbon nutrients from the petroleum hydrocarbons present in this sample. The results suggest that the application of oil-degrading commercial bacterial consortium accelerated the rate of CO_2 evolution and clearly increased biodegradation efficiency more than other treatments. The initial CO_2 evolution rate was shown to efficiently evaluate the treatability test by providing significant data within a short period, which is critical for the rapid determination of the appropriate bioremediation approach. The measurements of microbial activity and used lubricating oil degradation also validate the CO_2 evolution rate as an appropriate criterion.

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