

Dynamics of CO₂ 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 S₁ and sandy S₂) 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: Amnrite p1300 as the bioaugmentation (T₁), other treatments consist of nutrients amendments - (NH₄)₂SO₄ and K₂HPO₄ (NPK) as biostimulation (T₂), unammended soil - natural attenuation as (T₃) and the control soil treated with sodium azide (NaN₃) as (T₄) 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 CO₂ evolution, microbial activity, and oil degradation rate. In Soil 1, T₁ 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 (T₄) 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 (S₂) with the highest CO₂ production in T₁. The best percentage oil degradation was also recorded where the utmost CO₂ production was obtained.

Key words: *Bioremediation, Hydrocarbons, Oil-contaminated soil, Commercial bacterial, Carbon dioxide respiration, Used lubricating oil.*

1.0 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

38 optimized conditions through adequate oxygen supply and mixing, by adjusting temperature,
39 pH and water content, performing nutrients amendment (biostimulation) or adding a suitable
40 microbial population (bioaugmentation). Biodegradation of complex hydrocarbon usually
41 requires the cooperation of more than a single bacterial species. This is particularly true in
42 pollutants that are made up of many different compounds such as petroleum compounds and
43 complete mineralization to CO₂ and H₂O is desired. One of the greatest advantages on the
44 study of bioremediation in petroleum hydrocarbons contaminated soil treatment is its cost
45 effectiveness, as compared to some physicochemical techniques, which are expensive and
46 need continuous monitoring in order to attain successful results. Bioremediation has emerged
47 as a good technique for environmental treatment regarding organic compounds, such as
48 petroleum hydrocarbons, due to its flexibility and adaptability in different sites (Ryan, 1991).

49
50 Natural soils often contain the microorganisms necessary for degrading compounds formed in
51 nature (Alexander, 1994), but bioaugmentation of the populations may enhance the rate of
52 bioremediation. Furthermore, inorganic nutrient supplementation may speed up the process,
53 because the addition of large quantities of oil results in a high C:N ratio that is unfavourable
54 to microbial activity (Choi *et al.*, 2002). Activities of microorganism is essential to nutrient
55 cycling in soils, and any effect which pollution has on soil microorganisms will also affect
56 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
57 diversity and activity are rapidly altered by such perturbation (Schloter *et al.*, 2003). The
58 measurement of microbiological parameters, such as soil respiration, microbial biomass,
59 provides information on the presence and activity of viable microorganisms as well as on the
60 intensity, kind and duration of the effects of hydrocarbon pollution on soil metabolic activity;
61 such measurements may serve as a good index of the impact of pollution on soil health
62 (Brohon *et al.*, 2001; Eibes *et al.*, 2006). However, results on the effects of hydrocarbon
63 pollution on microbial biomass and activity are not always coincident, probably due to the
64 differences in chemical properties of the hydrocarbon used (Xu and Johnson, 1995).

66 A standard test in the initial assessment of biodegradation of any contaminant is the
67 measurement of evolved CO₂ (Sharabi and Bartha, 1993). Carbon (iv) oxide measurements
68 are simple, non-destructive, and representative of ultimate biodegradation. Most studies of the
69 biodegradation of organic contaminants have used CO₂ evolution as a measure of either the

microbial activity or of mineralization (Venosa *et al.*, 1992; Huesemann and Moore, 1993). The evolved CO₂, 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 CO₂ (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 CO₂ 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 CO₂ evolution as an index of mineralization of different contaminated with used lubricating oil hydrocarbons. This study also attempted to determine the relationships between CO₂ 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. Amnrite 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 colorimetry 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 $(25 \pm 1^{\circ}\text{C})$ 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 5×10^8 cfu/g of bran. In addition to bioaugmentation using microbial consortium, the polluted soils were amended with $(\text{NH}_4)_2\text{SO}_4$ 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

135 (NaN₃) (0.3% w/w) to inhibit soil microorganisms and to monitor abiotic hydrocarbon losses
136 on the microbial community in two different soil types. Microcosms were arranged in a
137 random order, and rearranged every 2 ± 2 weeks throughout the duration of the experiment.
138 Triplicates sample treatments were set up, the content of each container was tilled carefully
139 every week for aeration to take place, addition of sterile distilled water was added every week
140 throughout the 90 – day period to maintain moisture content at 70 % water holding capacity
141 (Pramer and Bartha, 1972).

142

143 2.3. Soil respiration

144 Amended-moist soil with water holding capacity (WHC) of approximately 70 % was
145 incubated in triplicate at $(25 \pm 1 ^\circ\text{C})$ under laboratory conditions, in one Litre (1L) glass flasks
146 sealed with teflon-lined rubber stoppers. Measurement of carbon (iv) oxide evolution was
147 performed through indirect method (Weytjens *et al.*, 1994). Microbial activity was monitored
148 by analyzing CO₂ accumulation in the headspace by gas chromatography. Headspace samples
149 of 1 cm³ were taken from microcosms with a Hamilton gastight® syringe and were injected
150 into a Gas Chromatograph Model GC- 14B (Shimadzu Crop Kyoto Japan), with a thermal
151 conductivity detector (TCD) at temperature of 150 ° C, injector at 100 ° C and Porapak -Q
152 column at 50 ° C, using nitrogen as a carrier gas. Respiration rate was expressed as evolved
153 CO₂ in mL CO₂ m⁻² h⁻¹, and the accumulated CO₂ concentration in mg CO₂/ kg of dried soil.
154 The amount of CO₂ evolved during the mineralization of used lubricating oil was monitored
155 using the accumulated concentration of CO₂ recorded from the CG-TCD. Soil samples were
156 withdrawn from each treatment for the residual analysis of Total Petroleum Hydrocarbons
157 (TPH) and Polycyclic Aromatic Hydrocarbons (PAHs) at every 15 days intervals to the end of
158 the experiment. Microcosms were aerated for 15 min after CO₂ measurement to maintain
159 oxygen levels in the system. Microcosms were set up in triplicate in tightly closed glass
160 flasks. To determine metabolic activity in each microcosm, respiration through CO₂ emission
161 monitoring were periodically performed. The respiration mean of the blanks was subtracted
162 from the treated microcosms, and the difference in CO₂ production between the blanks and
163 the treated microcosms was used as the amount of CO₂ produced.

164

165 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 K₂HPO₄, 4.0 g NH₄Cl, 0.2 g MgSO₄.7H₂O, 1.2 g KH₂PO₄, 0.01 g FeSO₄.7H₂O, 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.

179

180 **2.5. Statistical data analysis**

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

184 **3.0 Results and Discussion**

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

187 Table 1: Selected physical and chemical characteristics of the noncontaminated soil samples
188

189 Parameters	Soil 1 (S ₁)	Soil 2 (S ₂)
190 pH (H ₂ O)	5.20	4.92
191 Total Nitrogen (%)	0.43	0.11
192 Avail. P (mg/dm ³)	1.00	0.40
193 Organic C (%)	3.50	0.81
194 C:N ratio	8.14	7.56
195 Moisture Content (%)	33.80	11.30
196 Sand (dag/kg)	11.00	68.00

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

201

202 **3.1 Soil respiration (CO₂-evolution) analysis.** The values of CO₂ evolved during a
203 90-day incubation experiment from soil samples at different times in 3.0 % and 4.5 % level of
204 contamination are shown in Fig. 1 and 2 respectively. Dynamics of CO₂ emissions were
205 higher in the clayey soil than in the sandy soil both for the contaminated and the control
206 treatments. This occurrence might be as a result of the higher nutrient contents and microbial
207 metabolism in this clayey soil which has been shown to offer greater capacity for
208 physicochemical attenuation of contaminants than coarse sands. (Pye and Patrick, 1983).

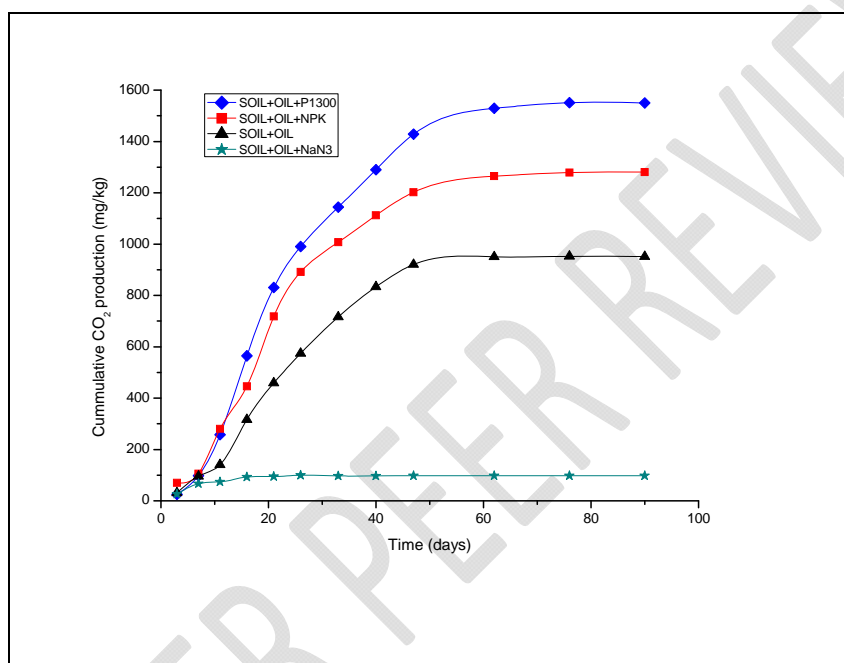
209 The contaminants at the two loading rates (3.0 and 4.5 %) assayed, increased soil
210 respiration especially in the clayey soil. The used lubricating oil in clayey contaminated soils
211 showed that respiration in 3.0 % loading rates producing the greatest CO₂ emissions of
212 (1600.20 mg/kg) and (1347.60 mg/kg) while the least values were recorded in the control (T₄)
213 with 89.52 and 102 mg/kg in soil contaminated with 3 and 4.5 % respectively. The same
214 noticeable trend was recorded in S2 where T1 produced the highest CO₂ production of 932
215 and 702 and the T4 recorded the least values of 113 and 64 mg/kg for 3 and 4.5 %
216 respectively. The high amount of CO₂ liberated in clayey soil amended T₁ is an indication of
217 high utilization of organic carbon nutrients from the petroleum hydrocarbons present in this
218 sample. The exception to this trends was that respiration rate of the samples with higher
219 contamination level of 4.5 % used lubricating oil was lower compared with the soils
220 contaminated with 3 %. The stimulatory effect of used lubricating oil on soil respiration
221 persisted in both soils throughout the incubation period, and was still noticeable 90 days after
222 contamination (Fig. 1 and 2). The cumulative evolution of CO₂ in the clayey soil (S1) at both
223 level of contamination (3.0 and 4.5 %) in the amended treatments with Amnrite p1300
224 products in (T1) were higher than treatments amended with nutrients (T2) in (3.0 and 4.5 %)
225 level of contamination. The lower values recorded in both soils contaminated with 4.5 %
226 might be due to the toxicity of the used lubricating oil to the microorganisms present in the
227 contaminated soils (Ijah and Antai, 2003; Rahman *et al.*, 2002). Soil respiration (in terms of
228 carbon dioxide - CO₂-evolution) in T1 and T2 were significantly higher than in T3 and T4.

229 The CO₂-evolution in T4 reached maximum values after 30 days, decreasing thereafter and
 230 remaining almost constant till the end of the 90-day experiment. Carbon dioxide (CO₂)
 231 evolution in T4 indicates that the sodium azide (biocide) used for this experiment was not 100
 232 % effective to inhibit the carbon oxidising bacterial metabolism during the experimental
 233 period.

234

235

S1



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237

238

S2

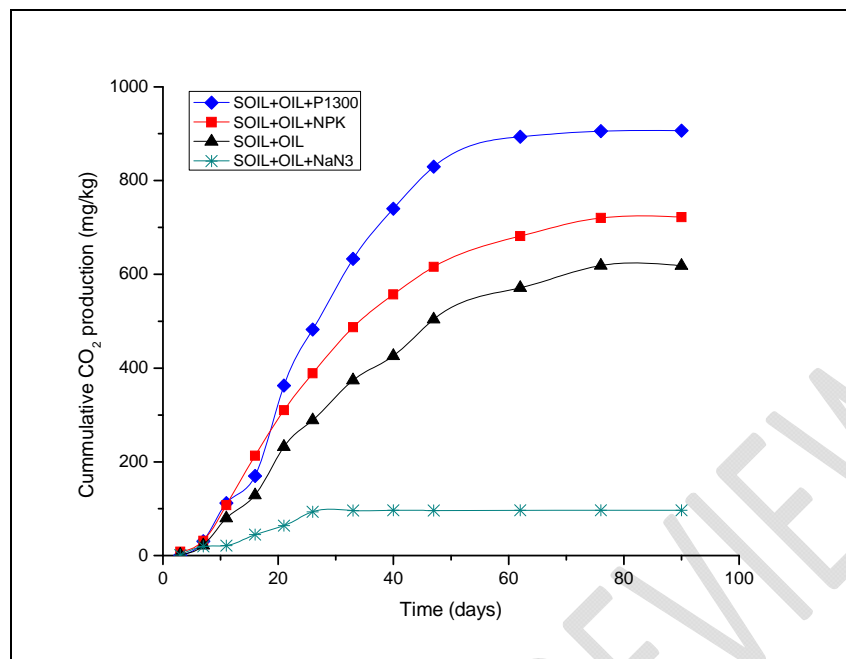
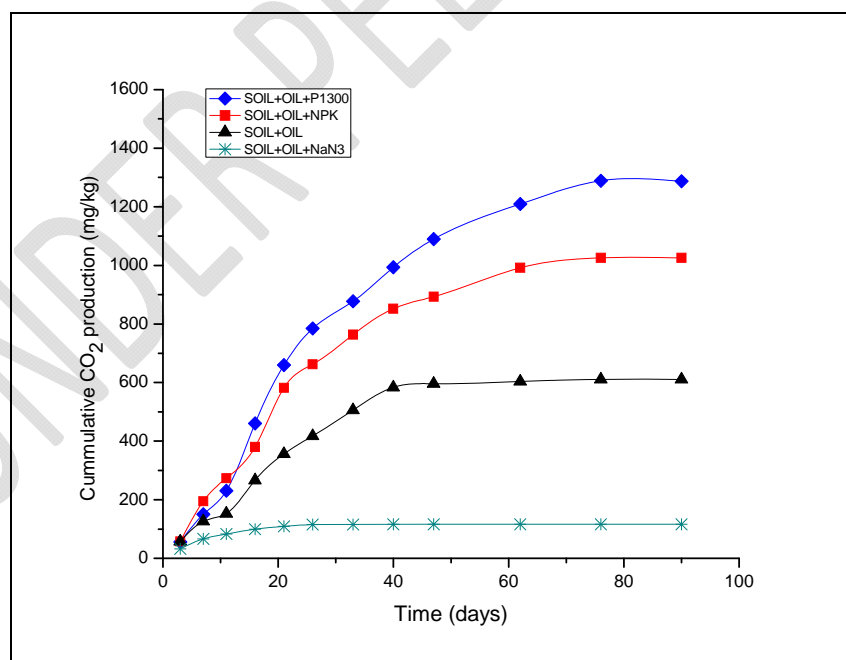


Fig. 1: Cumulative CO₂ production during biodegradation of clayey (S1) and sandy (S2) soils contaminated with 3 % used lubricating oil.

S1



S2

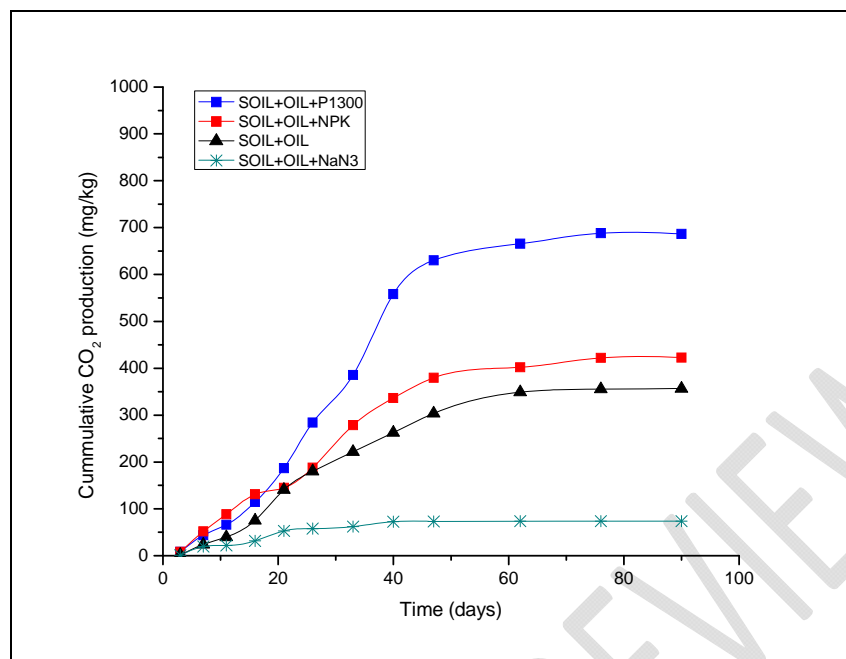
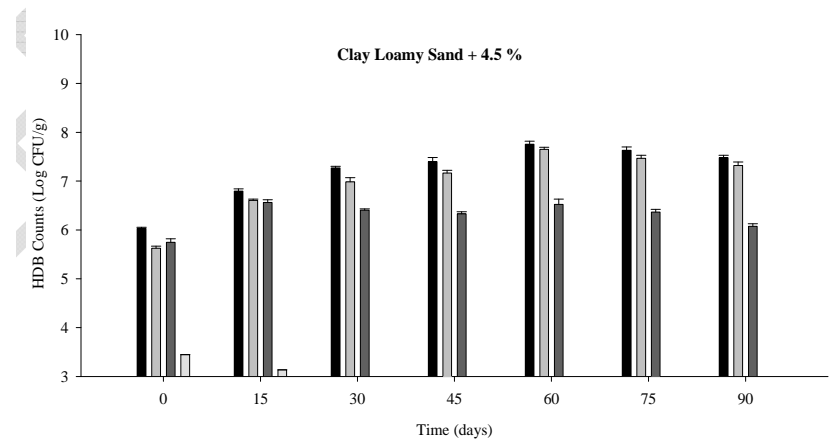
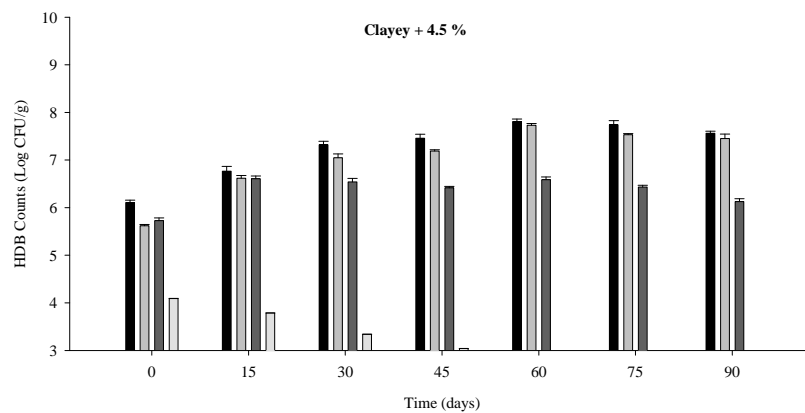
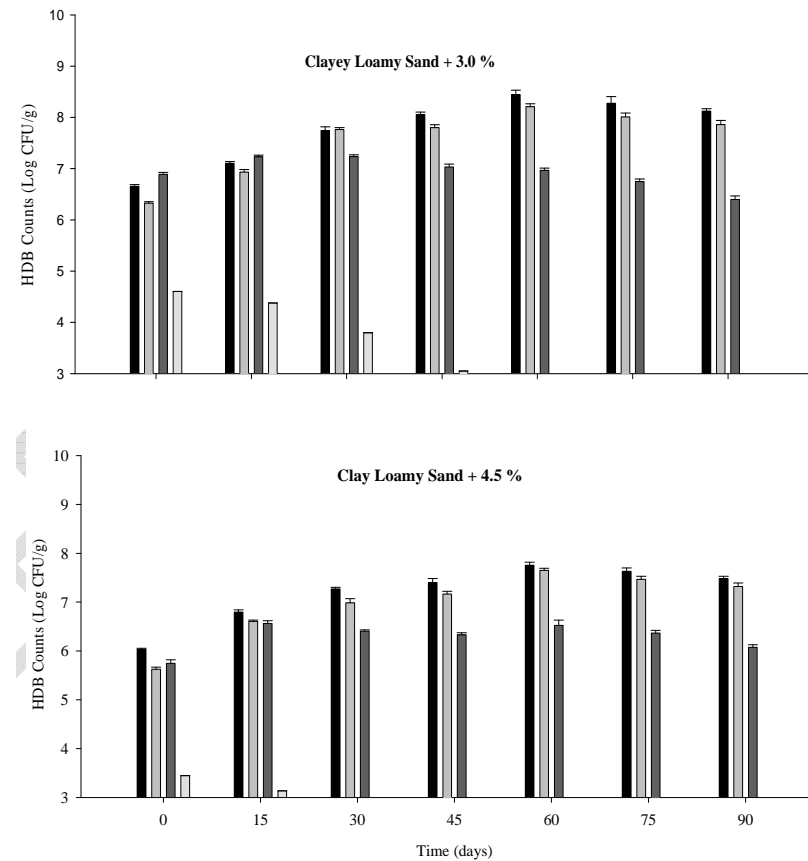
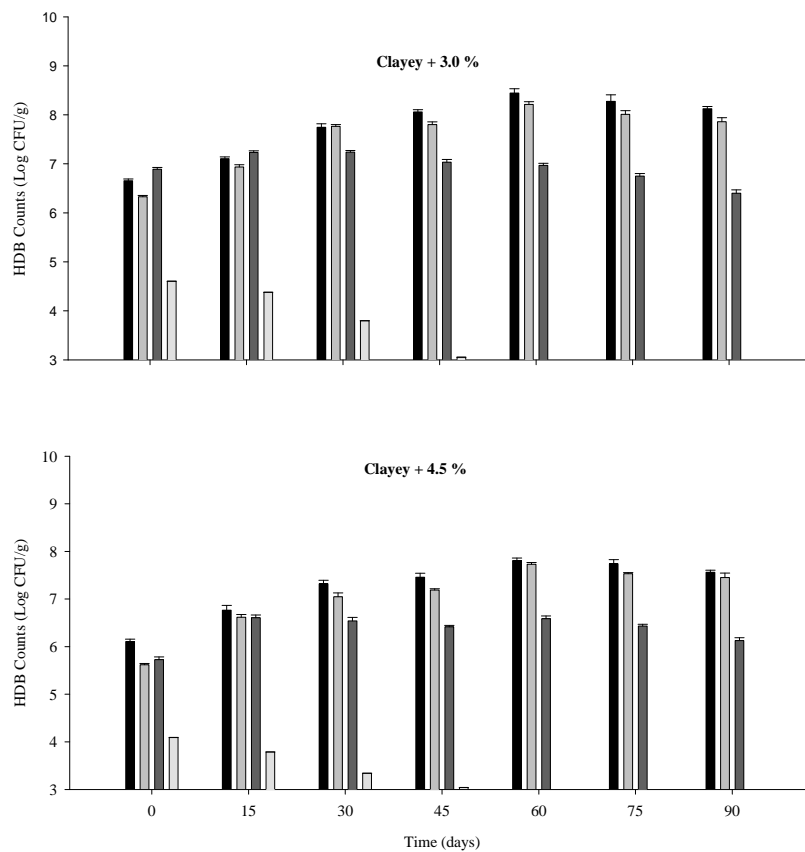


Fig. 2: Cumulative CO₂ production during biodegradation of clayey (S1) and sandy (S2) soils contaminated with 4.5 % used lubricating oil.

3.3. Enumeration of hydrocarbon degrading bacteria. The hydrocarbon degrading bacterial (HDB) counts in T1 contaminated with 3 % ranged from $(4.5 \times 10^6$ to 2.8×10^8 CFU/g) in S1 and $(4 \times 10^6$ 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 $(1.28 \times 10^6$ to 6.4×10^7 CFU/g) in S1 and $(1.08 \times 10^6$ 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 findings of Rahman *et al.* (2002) corroborated this that there was an increase in the rate of biodegradation of crude oil, as the concentration of oil reduced. 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).



■ SOIL+OIL+P1300 ■ SOIL+OIL+NPK
 ■ SOIL+OIL ■ SOIL+OIL+NaN3

■ SOIL+OIL+P1300 ■ SOIL+OIL+NPK
 ■ SOIL+OIL ■ SOIL+OIL+NaN3

Fig. 3: Hydrocarbon-degrading bacteria (HDB) counts in soils contaminated with 3.0 and 4.5 % used lubricating oil. Vertical bars 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 Amnate 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 Amnate 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 (Amnate 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

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

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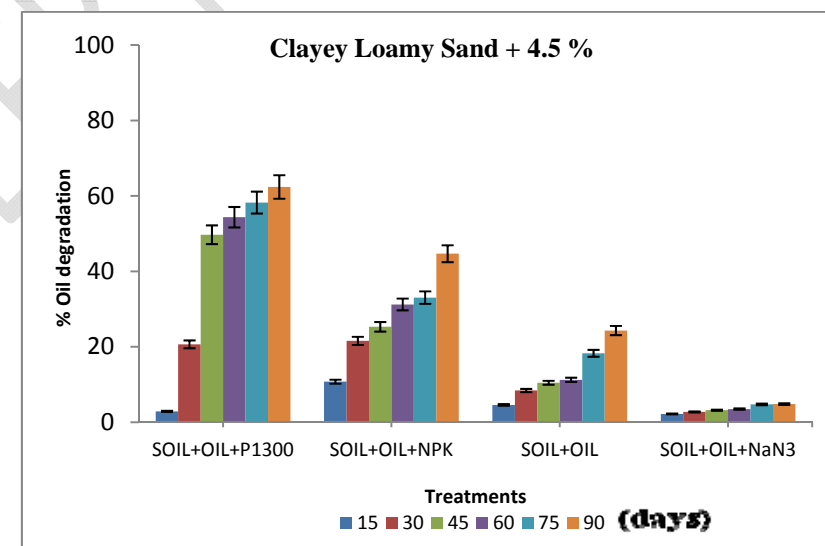
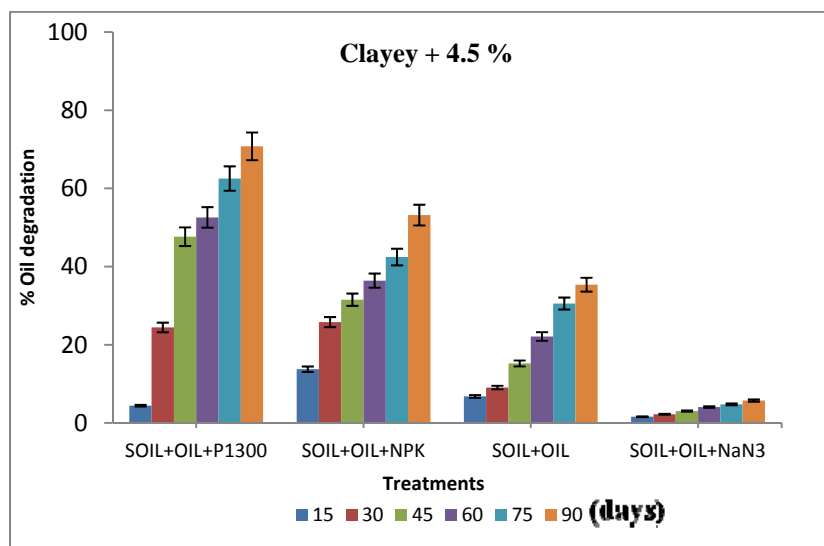
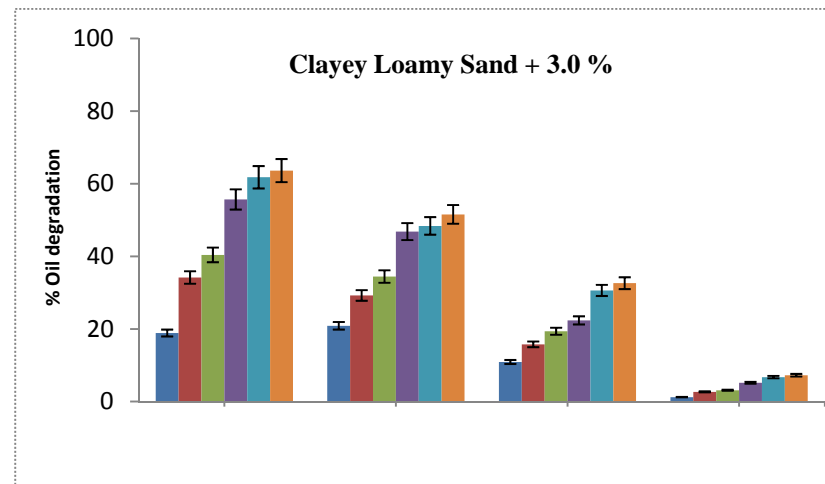
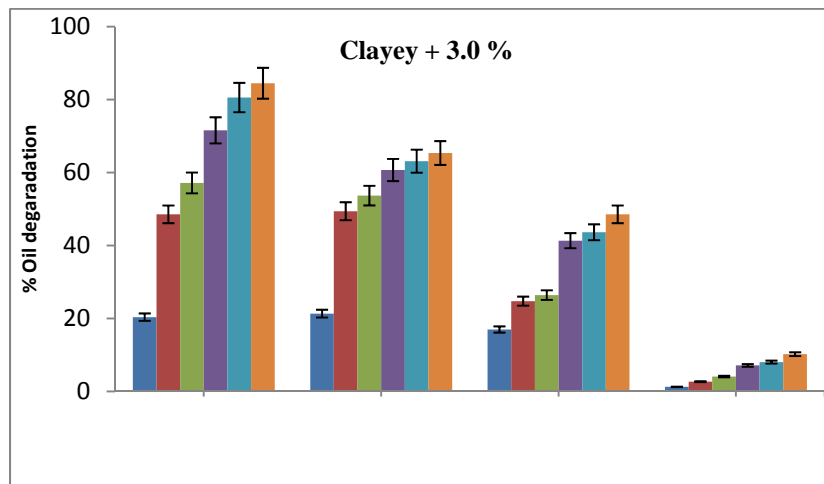


Figure 4: Percentage degradation of petroleum hydrocarbon in soil contaminated with 3.5 and 4.5% used lubricating oil in soil 1 (S1) and Soil 2 (S2). Vertical bars indicate standard error of the mean SE (n = 3).

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 CO₂ emissions. The higher amount of CO₂ liberated in clayey soil amended with amnrite 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 CO₂ evolution and clearly increased biodegradation efficiency more than other treatments. The initial CO₂ 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 CO₂ evolution rate as an appropriate criterion.

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