Original Research Article

BIOREMEDIATION OF THREE BRAZILIAN SOILS CONTAMINATED WITH USED LUBRICATING OIL

ABSTRACT

This study aimed at bioremediation potentials of organic pollutants, in particular, spent motor oil contaminated soils, using commercial microbial nutrient. Other objectives were the evaluation of kinetic model to determine the rate of biodegradation of petroleum hydrocarbon in soil and to subsequently determine the half-life of the oil degradation. The patterns of biodegradation of used motor oil were studied for a period of 90 days under laboratory condition. The model soil (300 g) was contaminated with 1.5 % (w/w) of used motor oil at room temperature in the laboratory using microcosm of 1 L. The microcosm was used to simulate the comparative effect of spent oil addition and bioremediation using a commercially available hydrocarbon degrading microbial consortium - Amnite p1300 as bioaugmentation (T1), nutrients amendments - $(NH_4)_2SO_4$ and K_2HPO_4 (NPK) as biostimulation (T2), unammended soil - natural attenuation as (T3) and the control soil treated with sodium azide (NaN_3) as (T4). Treatment effects were evaluated on microbial community using three soil types (S1, S2 and S3). Hydrocarbon-utilizing bacterial counts were obtained in the amended soils under treatments T1, T2, and T3 ranging from 3.47×10^6 to 3.27×10^8 cfu/g compared to T4 throughout the 90 days of study. Soils amended with Amnite p1300 showed highest reduction in total petroleum hydrocarbon with net loss of 36.17 % throughout the period of experiment compared to other treatments. The changes (decline and recovery) in population of microbial community are a useful and sensitive way of monitoring the impact and recovery of used motor oil-contaminated soils. The results suggest that different soils have different inherent microbial potential to degrade hydrocarbons of soils contaminated with spent oil.

Key words: Bioremediation used lubricating oil, hydrocarbons, microbial consortium, soil types.

1. Introduction

32

33 There is rise in consumption of automotive lubricating oil worldwide, this increases had
34 consequences for ecosystem health in terms of disposal of used engine/lubricating oil. In Brazil, the
35 problems tend to worsen with economic and population growth, and rapid industrialization without
36 concern thus disregard for environmental health, particularly in relation to used motor oil. The
37 consumption of lubricating oil in Brazil is around 10^6 m^3/year (Conselho em Revista, 2005; Magalhães
38 et al.,2007). Approximately 6.5 x 10^5 m^3/year are consumed in the lubrication process, and from 3.5 x
39 10^5 m^3/year remainder, only 20% are treated or recycled; therefore, significant volumes of used motor
40 oil are continually discharged into the ecosystems (local environment). Release of hydrocarbons into
41 the environment whether accidentally or due to human activities is a main cause of water and soil
42 pollution (Holliger et al., 1997). These hydrocarbon pollutants usually cause disruptions of natural
43 equilibrium between the living species and their natural environment. Despite efforts in some countries
44 to recover and recycle used motor oils, significant amounts of lubricants are input into the
45 environment, particularly in environmentally sensitive applications such as forestry and mining, or
46 through engine losses (Battersby, 2000). Consequently, considerable attention has been given to
47 lubricant biodegradability and persistence in the environment. Therefore, there is a need for effective
48 and environmentally safe cleanup treatments of oil spills (crude or used petroleum hydrocarbon
49 compounds). The United State Environmental Protection Agency (40 CFR Part 279) defined "used oil"
50 as "any oil that has been refined from crude oil or any synthetic oil that has been used and, as a result
51 of such use is contaminated by physical or chemical impurities." Used motor oil contains metals and
52 heavy polycyclic aromatic hydrocarbons derived from engine oil - a complex mixture of hydrocarbons
53 and other organic compounds, including some organometallic constituents (Butler and Mason, 1997)
54 that is used to lubricate parts of an automobile engine, in order to smooth engine operation (Hagwell
55 et al., 1992; Boonchan et al., 2000). The persistent hydrocarbon components are known to have
56 carcinogenic and neurotoxic activities (Reddy and Matthew, 2001; Das and Chandran, 2010). One
57 gallon of used motor oil, improperly disposed of, may contaminate 1 million gallons of fresh water,
58 which is enough to supply 50 people with drinking water for one year. One pint (4 gills or 568.26 cubic
59 centimetres) of used motor oil improperly disposed of can create a one-acre slick on the surface of a
60 body of water and kill floating aquatic organisms (USEPA, 1996).

61 Unsafe disposal of petroleum hydrocarbon products increase soil contamination, and this has
62 constituted major environmental problems. Therefore, the development of research and technologies
63 to remediate soils contaminated with used motor oils, in particular bioremediation, provides an
64 effective and efficient strategy to speed up the clean-up processes (Mandri and Lin, 2007). Various
65 factors including lack of essential nutrients such as nitrogen and phosphorus may limit the rate of
66 petroleum hydrocarbon degradation from contaminated soil. Addition of inorganic nutrients
67 (biostimulation) is therefore needed as an effective approach to enhance the bioremediation process

68   (Semple et al., 2006; Walworth et al., 2007). Also, many microbial strains, each capable of degrading

69   a specific compound, are available commercially for bioremediation (Bragg et al., 1994; Korda et al.,

70   1997; Song et al., 1990).

71

72   **2. MATERIAL AND METHODS**

73   **2.1. Collection of samples.** The soils used were collected from three locations in Minas

74   Gerais State, Brazil. Soils samples were collected in hermetic bags and transported to the laboratory

75   for analysis. Used engine oil was collected from a gasoline and car service station close to the Federal

76   University of Viçosa, Brazil. Amnite P1300 consisted of special bacterial strains (Amnite P1300)

77   specially made to degrade used motor oil was obtained from Cleveland Biotech Ltd., UK

78

79   **2.2. Experimental Design and set-up of microcosm.** Exactly 300 g each of the model

80   soils was contaminated with 1.5 % (w/w) or (15000 mg/kg) of used motor oil at room temperature (25 ±

81   1 ^{0}C) under laboratory conditions using 1 litre capacity microcosm. The microcosms were used to

82   simulate the biodegradation of effect of spent oil polluted soil using a commercially available

83   hydrocarbon degrading microbial consortium (Amnite P1300). Aminte consist of a mixture of *Bacillus*

84   *subtilis, Bacillus megaterium*, *Pseudomonas putida, Pseudomonas fluorescens, Phanerochaete*

85   *chrysosporium, Rhodococcus rhodocrous* on a cereal (bran) as the bioaugmentation treatment. The

86   microorganisms were conditioned to degrade heavy hydrocarbons. The total population of microbes in

87   Amnite P1300 was approximately 5 x 10^8 cfu/g of bran. Also, the polluted soils were amended with

88   $(NH_4)_2SO_4$ and K_2HPO_4) to simulate biostimulation. The C:N:P ratio of the nutrient compound was

89   adjusted to 100:7.5:1 (optimum conditions). The same conditions provided in the biostimulation

90   treatment were used in the bioaugmentation treatment in which the $(NH_4)_2SO_4$ and K_2HPO4 were

91   combined with p1300. The unammended soil (natural attenuation), in which nutrients were not added

92   while microbial inoculum was included to indicate hydrocarbon degradation capability of

93   microorganisms naturally present in the contaminated soils (i.e. the autochthonous microbes). There

94   was a control soil in which most of the indigenous bacteria were killed by the addition of a biocide,

95   sodium azide (NaN_3) (0.3% ww $^{-1}$) to inhibit soil microorganisms and to monitor abiotic hydrocarbon

96   losses on the microbial community in three different soil types. There were six sampling dates (15, 30,

97   45, 60 75 and 90); hence 36 microcosms in total were used. Microcosms were arranged in a random

98   order, and rearranged every 2 weeks ± 2 days throughout the duration of the experiment The

99   treatments were replicated 3 times, while the content of each container was tilled every week for

100 aeration, moisture content was maintained at 70% (Pramer and Bartha, 1972), and water holding

101 capacity by the addition of sterile distilled water every week until the end of the experiment.

102

103

104   **2.3. Sampling.** Periodic sampling from each microcosm was carried out at 15-day intervals
105   for 90 days. Composite samples were obtained by mixing 10 g of soil collected from different areas of
106   the microcosm for bacteria enumeration and determination of total petroleum hydrocarbon.
107
108   **2.4. Determination of the physicochemical property of the soi**l. Table 2 shows the
109   origin and selected physical and chemical characteristics of the non-contaminated soil samples used
110   for the bioremediation studies. Nitrogen content of the soil was determined using the Kjeldahl method,
111   the available phosphorus was determined by colometry after Mehlich 1 extraction and Organic Carbon
112   content was determined using a modifield Walkley-Black (1934) method. The pH was determined
113   using 1:2.5 ratio by weight with distilled water (w/v) after 30-min equilibration using a pH meter and
114   electrode calibrated with pH 4.0 and 7.0 standards. Determinations were made in triplicate.

115
116 Table 2: Selected physical and chemical characteristics of the non-contaminated soil samples

Parameters	Soil 1 (S$_1$)	Soil 2 (S$_2$)	Soil 3 (S$_3$)
pH (H$_2$0)	5.20	5.91	4.92
Total Nitrogen (%)	0.43	0.24	0.11
Avail. P (mg/dm^3)	1.00	1.8	0.40
Organic C (dag/kg)	3.50	1.54	0.81
C:N ratio	8.14	6.42	7.56
ECEC (cmol$_c$/dm^3)	3.19	2.29	0.78
Moisture Content (%)	33.80	28.3	11.30
Sand (dag/kg)	11.00	10.00	68.00
Silt (dag/kg)	9.00	22	4.00
Clay (dag/kg)	80.00	68	28.00
Texture	Clayey	Clayey	Clay loamy sand
Soil Type	Red Latosol	Red Lotosol	Red Yellowish Latosol
Parent Material	Sete Lagoas – MG	Cachoeira Dourada - MG	Tres Marias - MG

131

132

133 **2.5. Microbial monitoring and enumeration of total aerobic heterotrophic and**
134 **hydrocarbon-degrading bacteria.** Samples were collected fortnightly (0, 15, 30, 45, 60, 75, 90
135 days) over the entire period of study of the variously amended soils (S$_1$, S$_2$ and S$_3$). In order to monitor
136 cell numbers and biodegradation, 1 g of soil was removed from each microcosm at the set times and
137 suspended in 9 mL of saline solution in sterile centrifuge tubes. The mixture was vigorously shaken on
138 a vortex mixer for 3 minutes and then the soil particulates were allowed to settle for 1 min before 0.1
139 mL of the supernatant was sampled for CFU counts. The number of colony-forming total aerobic
140 heterotrophic bacteria (AHB) was determined by plating three replicate samples from each treatment
141 withdrawn every 15 days. Serially diluted samples (0.1 mL) were plated on nutrient agar medium
142 (Oxoid) supplemented with 10 mg/mL solution of cycloheximide in which 1 mL/L was drawn to
143 suppress the growth of fungi. The oil agar plates were incubated at 30°C for 24 hours, and the
144 colonies were counted. Also, enumeration of hydrocarbon-degrading bacteria (HDB) was attempted on
145 a mineral medium containing motor oil as the sole carbon source. The mineral medium contained 1.8
146 g K$_2$HPO$_4$, 4.0 g NH$_4$Cl, 0.2 g MgSO$_4$.7H$_2$O, 1.2 g KH$_2$PO$_4$, 0.01 g FeSO$_4$.7H$_2$O, 0.1 g NaCl, 20 g
147 agar, one percent (1%) used engine oil in 1,000 mL distilled water, and the medium was adjusted to

148   pH 7.4 (Zajic and Supplission, 1972). The oil agar plates were incubated at 30 ℃ for 7 days before the

149   colonies were counted.

150

151   **2.6. Extraction of residual oil and analysis of total petroleum hydrocarbons.**

152   Total Petroleum Hydrocarbons (TPHs) were extracted according to EPA method 3546 (US EPA, 2007)

153   using the Microwave Automated Reaction System from CEM (Matthews, NC). Briefly, Sodium sulfate

154   (Na_2SO_4) was purified by drying overnight in an oven at 150 ℃ and quickly transferred into a

155   desiccators. Five grams (5 g) of homogenised contaminated soil was weighed out, mixed with 5 g dry

156   anhydrous Na_2SO_4 and ground to less than 1 mm particle sizes, extracted in GreenChem vessels with

157   25 mL of a 1:1 hexane:acetone mixture according to manufacturer's protocol at 100 ℃ for 20 minutes.

158   The n-hexane and acetone was filtered through whatman No 1 filter paper to separate the extract from

159   the soil particles, and transferred into 100 mL amber vials through separatory funnel and sequentially

160   rinsed with equal volume of solvent mixture. The solvent were evaporated to partial dryness with a

161   rotary evaporator (Fizatom Rotavapor 801), transferred into 2 mL vials and then dried completely

162   using nitrogen gas. Dried samples were dissolved in 600 µL dichloromethane for gas chromatography

163   analysis. The residual oil was analyzed on Shimadzu GC-17A Chromatograph equipped with a Flame-

164   Ionization Detector (FID) by using fused silica capillary column DB-5 (30 x 0.25 mm), and AOC-17

165   Shimadzu auto injector complying with Environmental Protection Agency (EPA) standard method 8015

166   (US EPA 2007). The flow rate of the helium carrier gas was 1.81 mL/min with linear velocity of 38.49

167   cm/s. The initial temperature was programmed at 40^oC and held for 15 min. The temperature was then

168   increased to 280^oC at a rate of 10^oC /min. The final temperature was held for 31 min. The injector was

169   set in the split mode, the split ratio was set to 1:10; the injection volume was 1 µL and the injector and

170   the detector temperature for GC were maintained at 260 and 280 ℃, respectively, and the oven

171   temperature was programmed to rise from 40 to 280 ℃ in 10 ℃/min increments and to hold at 280 ℃

172   for 31 min. The dry weight of the soil samples was determined following baking of 10 g of wet soil at >

173   80 ℃ for at least 48 hours. Before analyzing the sample extract, a mixture of standards including *n*-

174   alkanes (*n*-decane *n*-C_{10}, *n*-dodecane *n*-C_{12}, *n*-tetradecane *n*-C_{14}, *n*-hexadecane *n*-C_{16}, *n*-octadecane

175   *n*-C_{18}, *n*-eicosane n-C_{20}, *n*-docosane *n*-C_{22}, *n*-tetracosane *n*-C_{24}, *n*-hexacosane *n*-C_{26}, *n*-octacosane *n*-

176   C_{28} and a pure standards containing *n*-triacontane *n*-C_{30}, *n*-dotriacontane *n*-C_{32}, *n*-tetratriacontane *n*-

177   C34, and *n*-hexatriacontane *n*-C_{36}, and a mixture of polycyclic aromatic hydrocarbon consisting of

178   acenaphthene, acenaphthylene, anthracene, benzo (a) anthracene, benzo (a) pyrene, benzo (b)

179   fluoranthene, benzo (g, h, i) perylene, benzo (k) fluoranthene, chrysene, dibenz (a, h) anthracene,

180   fluoranthene,fluorine, indeno (1, 2, 3-cd) pyrene, naphthalene, phenanthrene, pyrene, 1-

181   methylnaphthalene and 2-methylnaphthalene, Supelco) were used for calibration. Five points

182   calibration curves using peak areas were obtained and the response factors were used to determine

183   the concentrations of various hydrocarbons in the sample extract. The total petroleum hydrocarbons

184   were identified and quantified by comparing the peak area of samples with that of the standard of the

185 TPH mixture with reference to the curve derived from standards. Percentage of degradation was
186 calculated by the following expression:

$$\% \ biodegradation \ = \left[\frac{TPH \ control \ – \ TPH \ treatment}{TPH \ control}\right] \times 100 \qquad (1)$$

187

188 TPH data were fitted to the first-order kinetics model (Yeung et al., 1997):

$$y = a \ e^{-kt} \qquad (2)$$

189 where y is the residual hydrocarbon content in soil (mg/kg), a is the initial hydrocarbon content in soil
190 (mg/kg), k is the biodegradation rate constant (day^{-1}) and t is time (days). The biodegradation rate
191 constant *(k),* and half-life *ln(2)/k* of the hydrocarbons in soil during the bioremediation process were
192 calculated from the model using Statistical ® software (Stat Soft. Inc. (2007). The model was used to
193 estimate the rate of biodegradation and half-life of hydrocarbons in soil under each treatment and the
194 model was based on the assumption that the degradation rate of hydrocarbons positively correlated
195 with the hydrocarbon pool size in the soil.

196

197 **2.7. Statistical Analysis.** Statistical analysis of data obtained was carried out using
198 analysis of variance. Means of different treatments were also compared statistically using a General
199 Linear Model (ANOVA) (Tukey test, P >0.05) using statistical 8.0 software (Stat Soft, 2007).

200
201 **3. RESULTS AND DISCUSSIONS**
202

203 **3.1. Microbial Counts**. The aerobic heterotrophic bacterial (AHB) counts in T1 ranged between
204 1.01 x 10^8 and 2.4 x 10^9 CFU/g while T2 and T3 ranged from 1.03 x 10^8 to 1.7 x 10^9 and 1.0 x 10^8 to
205 3.8 x 10^8 CFU/g respectively (Fig. 3) across soil types. The treatment T4 had AHB counts ranging
206 from 1.27 x 10^3 to 6.03 x 10^5 CFU/g. Hydrocarbon Degrading Bacterial (HDB) counts were also higher
207 in spent oil contaminated soil under T1, T2 and T3 (Fig. 4). The count of HDB in soil amended with
208 Amnite p1300 (T1) was about 2% higher than those amended with $(NH_4)_2SO_4$ and K_2HPO_4 (T2) and
209 unamended – natural attenuation (T3). HDB count in soil amended with T1 ranged from 3.6 x 10^6 to
210 3.3 x 10^8 CFU/g, while those amended with T2 and T3 ranged from 3.7 x 10^6 to 2.6 x 10^8 and 3.5 x 10^6
211 to 5.41 x 10^7CFU/g, respectively. However, the HDB counts in T4 lower than T1, T2 and T3 ranged
212 from 1.07 x 10^3 to 7.07 x 10^4 CFU/g. These results were similar to that obtained by Antai and Mgbomo
213 (1989) whose counts of HDB in hydrocarbon-contaminated soil was 10^8 CFU/g, but higher than that of
214 Ghazali et al. (2004), who obtained 10^7CFU/g; from hydrocarbons degradation in diesel oil polluted
215 soil. The discrepancies in the results may be due to the characteristics from different ecologies of the
216 different soil types used for the experiments. The microbial counts of the high clayey soil (S1) and low
217 clay soil (S2) were similar in HDB. Counts in soils amended with T1 were highest followed by T2 and
218 T3. Whereas, microbial counts in Clay loamy sand (S3) showed different pattern compared with S1

7

219   and S2. Sodium azide (NaN_3) treated soil (T4) has the least results in all the soils used for the
220   experiment. This result clearly demonstrates the benefit of bioaugmentation, biostimulation and
221   indigenous microorganisms from used lubricating oil polluted soil. The different responses of the
222   investigated are shown in Figure 3 and 4, T4 is a control system where most of the indigenous
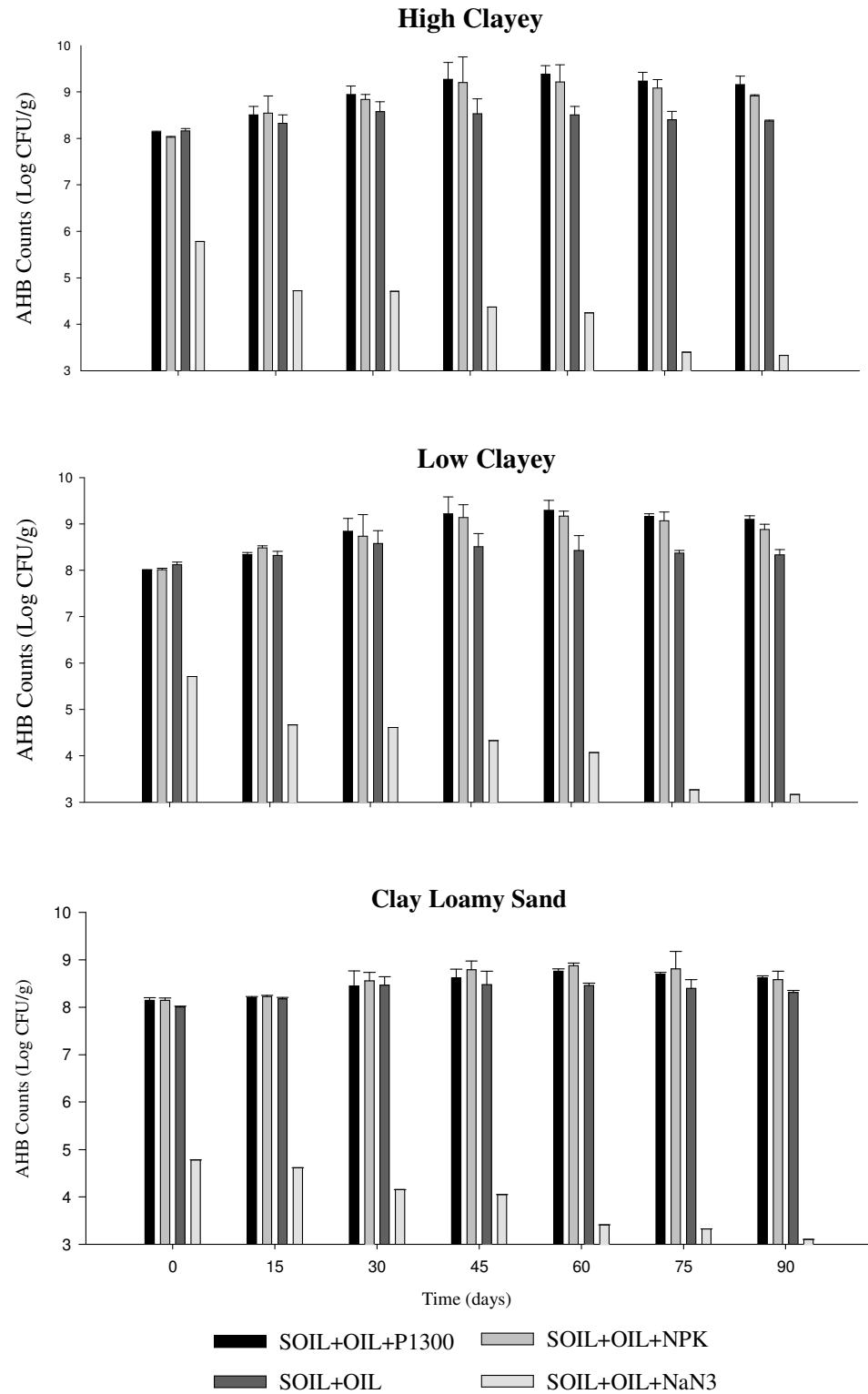223   bacteria were killed with a biocide (NaN_3).
224
225

Fig 3: Counts of aerobic heterotrophic bacterial (AHB) population in oil-contaminated soils. Vertical bars indicate standard error of means SE (n=3)
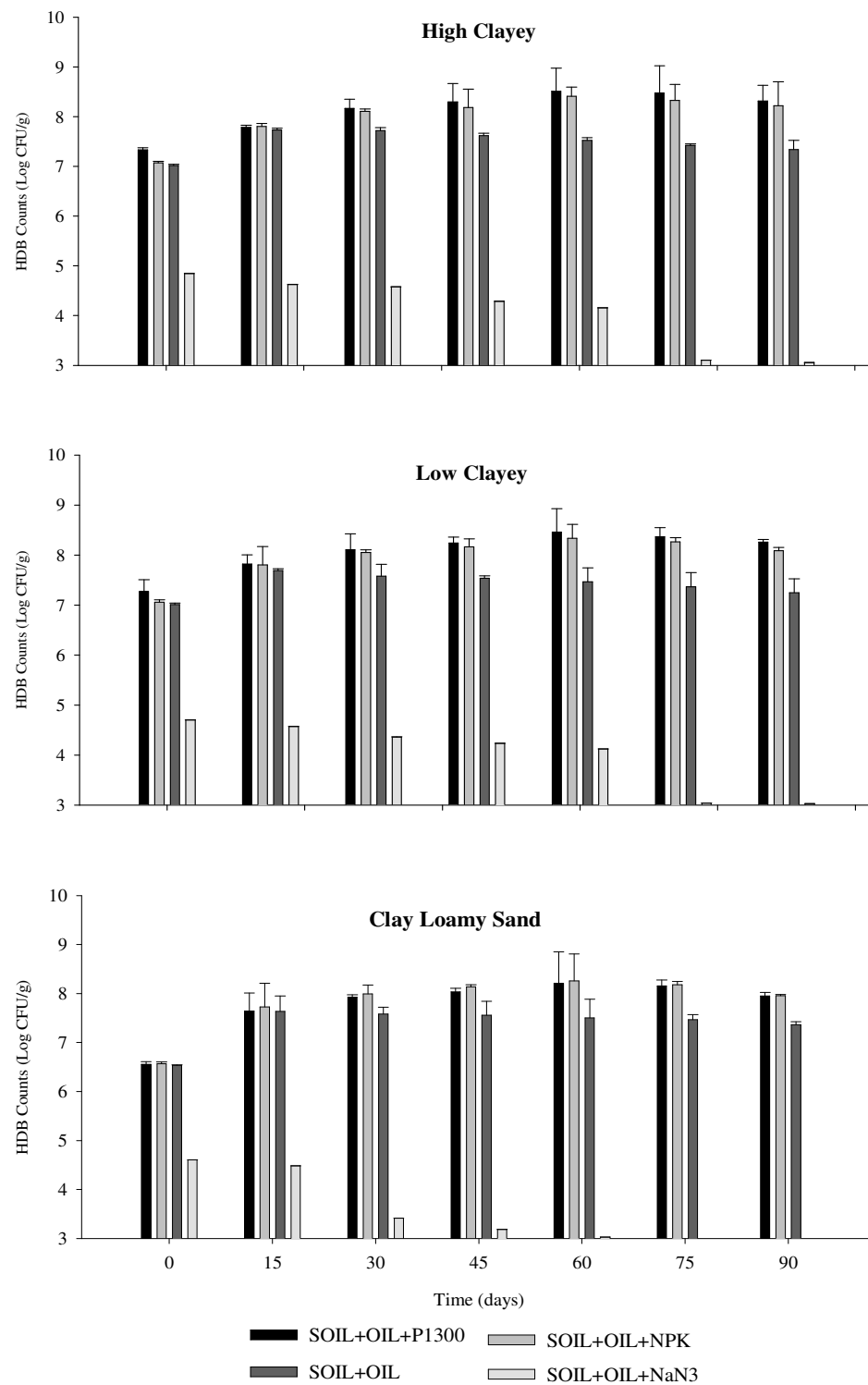
231

232

233
234 Fig 4: Counts of hydrocarbon degrading bacterial (HDB) population in oil-
235 contaminated soils. Vertical bars indicate standard error of means SE (n=3)
236

237

238   **3.2. Used engine oil hydrocarbon biodegradation.** There was a noticeable reduction
239   in the total petroleum hydrocarbon within the first 15 days in all the treatments, but higher reduction
240   was observed at 30 days for T1, T2 and T3 compared to the control (T4). At the end of 30 days, 49, 69
241   and 73 % TPH reduction were obtained in T3, T1 and T2 respectively. About 7,306; 10,278 and
242   10,881 mg/ kg reduction in TPH was observed in these treatments compared to 27 % (3,991 mg/ kg)
243   TPH reduction in the control soil (S1). Similar trend was noticed in soils S2 and S3 with T2 (NPK)
244   having the highest TPH reduction (Fig. 5). Because, feeding nutrient solutions containing inorganic
245   substances, such as nitrogen and phosphorus to natural soil bacteria population often enhances the
246   ability of the microorganisms to degrade organic molecules into carbon dioxide and water (Stotzky and
247   Norman, 1961a; and b). During this period, the added bacteria product acclimatized to their new
248   source of carbon. At the end of (90 days), oil-contaminated soil amended with T1 (Soil + Oil + Amnite
249   P1300) showed the highest reduction in soil concentration of used engine oil (89%), followed closely
250   by soil amended with T2 (Soil + Oil + NPK) (78%), but no significant differences were observed
251   between the treatment T1 and T2. Lower reduction in TPH obtained in soil type S3 compared to S1
252   and S2, may be due to high clay content in these soils which have been shown to offer greater
253   capacity for physicochemical attenuation of contaminants than coarse sands (Pye and Patrick, 1983).
254   However, highest reduction (68%) of TPH was observed in soil amended with T2 in soil S3 at the end
255   of 90 days experiments. The net percentage loss of used oil in the contaminated soils could indicate
256   the effectiveness of the treatments in biodegradation. The highest net percentage loss was observed
257   at 30 days in T2 (45.93%), (40.33%) and (32.58%) followed by T1 (41.91%), (36.36%) and (28.83%)
258   and T3 (22.10%), (22.10%) and (10.32%) in soils S1, S2 and S3, respectively (Table 3). However, the
259   net percentage loss of used oil increased from45 days in T1 to the end of the experiment (90 days)
260   compared with other treatments.

261 Table 3: Net percentage loss of total petroleum hydrocarbon in soils during bioremediation
262
263

Soil types	Treatments	Time (days)					
		15	30	45	60	75	90
S1	T1	18.53±1.3	41.91±1.4	29.59±0.7	33.50±1.2	34.56±1.0	36.17±0.8
	T2	20.54±1.4	45.93±1.3	29.37±0.8	28.28±0.7	26.88±1.2	25.94±0.8
	T3	7.08±1.3	22.10±1.4	18.10±0.7	17.08±0.4	15.77±1.2	16.13±0.8
S2	T1	15.29±1.0	36.36±0.4	34.21±1.0	31.54±1.7	32.31±1.3	31.66±2.3
	T2	17.21±2.0	40.33±1.2	33.68±0.5	31.19±1.0	31.37±1.2	23.47±2.1
	T3	4.86±1.7	20.77±1.1	15.24±1.9	12.90±1.4	13.48±1.2	10.58±2.1
S3	T1	10.54±2.6	28.83±2.3	21.21±1.8	22.48±2.6	23.59±0.9	23.47±2.1
	T2	10.84±1.1	32.58±1.4	22.88±1.0	23.50±0.8	24.53±0.9	24.99±2.3
	T3	4.86±2.2	10.32±0.4	6.77±0.8	6.24±1.4	5.70±0.8	10.58±2.1

285 T1=soil+oil+Amnite P1300, T2=soil+oil+($(NH_4)_2SO_4$ and K_2HPO_4), T3=soil+oil alone; S1=Red Latosol, S2 = Red Latosol, S3= Red-
286 Yellowish Latosol; Net % loss = % loss in TPH of oil-contaminated amended soils and oil-contaminated soil alone – % loss in TPH
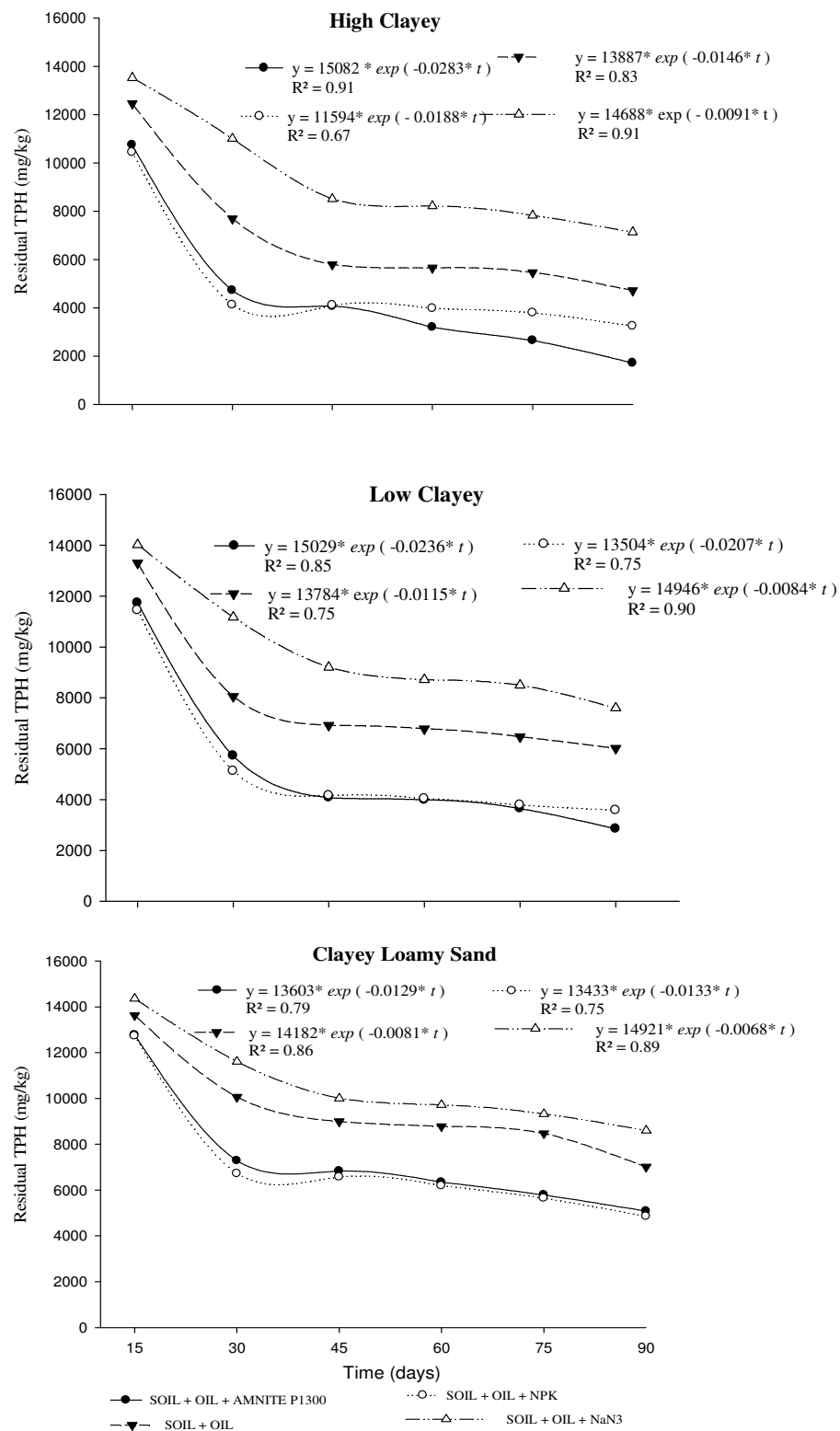287 of unamended contaminated control soil with sodium azide.

High Clayey

$y = 15082 * exp (-0.0283* t)$
$R^2 = 0.91$

$y = 13887* exp (-0.0146* t)$
$R^2 = 0.83$

$y = 11594* exp (- 0.0188* t)$
$R^2 = 0.67$

$y = 14688* exp (- 0.0091* t)$
$R^2 = 0.91$

294

Low Clayey

$y = 15029* exp (-0.0236* t)$
$R^2 = 0.85$

$y = 13504* exp (-0.0207* t)$
$R^2 = 0.75$

$y = 13784* exp (-0.0115* t)$
$R^2 = 0.75$

$y = 14946* exp (-0.0084* t)$
$R^2 = 0.90$

295

Clayey Loamy Sand

$y = 13603* exp (-0.0129* t)$
$R^2 = 0.79$

$y = 13433* exp (-0.0133* t)$
$R^2 = 0.75$

$y = 14182* exp (-0.0081* t)$
$R^2 = 0.86$

$y = 14921* exp (-0.0068* t)$
$R^2 = 0.89$

Time (days)

SOIL + OIL + AMNITE P1300   SOIL + OIL + NPK

SOIL + OIL   SOIL + OIL + NaN3

296

297   Fig 5. Residual total petroleum hydrocarbons in soils during bioremediation

13

3.3. Biodegradation kinetics (rate constant and half-life). The highest biodegradation rates of 0.0283, 0.0236 and 0.0133 day^{-1} and half-lives of 24.49, 29.37 and 52.12 days were recorded under amnite p1300 in soil types S1 and S2 and nutrient amendment in soil S3, respectively. The control T4 showed the least biodegradation rate of 0.0091, 0.0084 and 0.0068 with highest half-lives of 76.17, 82.52 and 101.93 in soils S1, S2 and S3 respectively. The biodegradation rate obtained under amnite amendment of used oil T1 showed the best result for the kinetic parameters in this study, as a result of the added bacterial products, followed by T2 and T3, and this may be due to the bioavailability of the inorganic nutrients to the indigenous bacterial population present in the soils (Table 4).

Table 4: Biodegradation rate constant (K) and half-life (t $_{0.5}$) of hydrocarbon in oil-contaminated soils

Treatments	K (day^{-1})			t $_{0.5}$ (days)		
—	S1	S2	S3	S1	S2	S3
T1	0.0283 Dc	0.0236Cb	0.0129 Ca	24.49 Aa	29.37 Ab	53.73 Ac
T2	0.0188Cc	0.0207 Cb	0.0133Da	36.87 Aa	33.49 Ab	52.12 Ac
T3	0.0146 Bc	0.0115 Bb	0.0081 Ba	47.48 Ba	60.27 Bb	85.57 Bc
T4	0.0091Ac	0.0084 Ab	0.0068 Aa	76.17 Ca	82.52 Cb	101.93 Cc

T1=soil + oil + Amnite P1300, T2= soil + oil + (NH$_4$)$_2$SO$_4$ and K$_2$HPO$_4$), T3 = soil + oil alone, T4 = unamended contaminated control soil with sodium azide. S1 = Red Latosol, S2 = Red latosol, S3 = Red-Yellowish Latosol; K = Biodegradation constant (day^{-1}) and H = Half life (days). Values followed by the same capital or small letters are not significant difference between treatments (column) or soil types (row) respectively at the P < 0.05 level, while values followed by different capital or small letters indicate significant differences between treatments (column) or soil types (row) respectively at the P < 0.05 level.

343   **4. CONCLUSIONS**

344

345   Hydrocarbon-degrading bacteria counts were higher ranging from 3.47×10^6 to 3.27×10^8 CFU/g

346   in the amended soils under treatment T1, T2 and T3 compared to T4 throughout the 90 days of study.

347   Spent engine oil contaminated soil amended with amnite (T1) showed the highest reduction in total

348   petroleum hydrocarbon with net loss of 36.17 % throughout the 90 days of the experiment compared to

349   other treatments. The changes in population of microbial community (decline and recovery) are useful

350   and sensitive means of monitoring the degradation and recovery of used lubricating oil-contaminated

351   soils. Commercially available microbial-based bioremediation products appeared to be promising in the

352   removal of petroleum hydrocarbons from contaminated clayey soil.

353   The tested kinetic model of biodegradation showed the highest biodegradation rate of 0.0283

354   day-1 and least half life of 24.49 days of the spent oil contaminated soil biodegradation was highest in

355   high clayey and soil organic matter contents. This reveals the influence of organic matter in the

356   degradation of petroleum hydrocarbons contaminated soils.

357   Remediation of hydrocarbons contaminated soil is necessary in order to preserve the safety and

358   health of the ecosystem with consequences on environmental and human health. Biological remediation

359   of hydrocarbon contaminated soil offers a better and more environmentally friendly technique that should

360   be properly due to its enormous advantages over other methods of remediation. However, despite these

361   enormous advantages of bioremediation, its potential is yet to be fully utilized in restoration of

362   contaminated soil. This is possibly due to the fact that it takes a long period of time for the complete

363   restoration of contaminated soil. This limitation can however be overcome through nutrient addition and

364   introduction of microbes with biodegradative capability on petroleum hydrocarbons.

365

372

373

374

375

376

5. REFERENCES

377

378 Antai, S. P., and Mgbomo, E. (1989). Distribution of hydrocarbon utilizing bacteria in oil-spil areas.
379   Microbiology Letters, 40, 137–143.

380 Battersby NS (2000) The   biodegradability and microbial toxicity testing of lubricants – some
381   recommendations. Chemosphere 41, 1011–1027
382

383 Bragg, J. R., R. C. Prince, J. B. Wilkinson, and R. M. Atlas. (1994). Effectiveness of bioremediation for the
384   *Exxon Valdez* oil spill. Nature 368, 413–418.
385

386 Butler, C. S., and Mason, J. R. (1997). Structure–function analysis of the bacterial aromatic ring–
387   hydroxylating dioxygenases. Advanced Microbial Physiology, 38, 47–84.
388

389 Boonchan, S., Britz, M. L., & Stanley, G. A. (2000). Degradation and mineralization of high-molecular
390   weight polycyclic aromatic hydrocarbons by defined fungal– bacterial co-cultures. Applied
391   Environmental Microbiology, 66(3), 1007–1019
392

393 Conselho em Revista.   (2005). Conselho Regional de engenharia, arquitetura e agronomia do Rio
394   Grande do Sul. Mais rigor no controle de lubrificante.Ano II, v.13,p.11–13.
395

396 Das, N. and P. Chandran, (2011). Microbial degradation of petroleum hydrocarbon contaminants: An
397   overview. Biotechnol. Res. Int., 2011, 1-13.
398

399 Ghazali F; Rahman R; and Basri A. (2004) Biodegradation of hydrocarbons in soil by microbial
400   consortium. International Biodeterioration & Biodegradation 54, 61 – 67.
401

402 Hagwell, I. S., Delfino, L. M., and Rao, J. J. (1992). Partitioning of polycyclic aromatic hydrocarbons from
403   oil into water. Environmental Science and Technology, 26, 2104–2110.
404

405 Holliger, C., Gaspard, S., Glod, G., Heijman, C., Schumacher, W., Schwarzenbach, R.P., Vazquez, F.
406   (1997) Contaminated environment in the subsurface and bioremediation: Organic contaminants.
407   FEMS Microbiology Reviews 20(3-4), 517 – 523.
408

409 Korda, A., P. Santas, A. Tenete, and R. Santas. (1997). Petroleum hydrocarbon bioremediation: sampling
410   and analytical techniques, *in situ* treatments and commercial microorganisms currently used.
411   Appl. Microbiol. Biotechnol. 48, 677–686.
412

413 Magalhães, J. M.; Donoso, F. F.; Melo, P. L .R. Re-refino no Brasil: A reciclagem de óleos usados. .Net,
414   Universidade   do   Vale   do   Rio   do   Sinos.   Disponível   em:
415   http://www.ead.fea.usp.br/semead/10semead/sistema/resultado/trabalhosPDF/584.pdf>. Acesso
416   em: 30 de nov. 2007.
417

418 Mandri, T., and Lin, J. (2007). Isolation and characterization of engine oil degrading indigenous
419   microorganisms in Kwazulu-Natal, South Africa. African Journal of Biotechnology, 6(1), 23–27.
420

421 Pye, V.I. and Patrick, R. (1983). Ground water contamination in the United States. *Science.* 221:713–718.

422   Reddy, C.A. and Z. Mathew, (2001). Bioremediation Potential of White Rot Fungi. In: Fungi in
423   Bioremediation, Gadd, G.M. (Ed.). Cambridge University Press, UK., ISBN: 0521781191, pp: 1-
424   12.

425   Semple, K. T., Dew, N. M., Doick, K. J., and Rhodes, A. H. (2006). Can mineralization be used to
426   estimate microbial availability of organic contaminants in soil? Environmental Pollution, 140, 164–
427   172.
428
429   Song, H.-G., X. Wang, and R. Bartha. (1990). Bioremediation potential of terrestrial fuel spills. Appl.
430   Environ. Microbiol. 56, 652–656.
431
432   StatSoft, Inc. (2007). STATISTICA (data analysis software system), version 8.0.
433
434   Stotzky, G. and Norman, A.G. (1961a). *Arch. Mikrobiol.* 40, 341–369.
435
436   Stotzky, G. and Norman, A.G. (1961b). *Arch. Mikrobiol.* 40, 370–382.

437   US EPA. (1996). Recycling used oil: What can you do? Cooperation Extension Services
438   ENRI, 317, 1–2.
439
440   US EPA (2007). United States Environmental Protection Agency), Method 3546, Microwave Extraction,
441   in: Test Methods for Evaluating Solid Waste, Physical Chemical Methods (SW–846), EPA, Office
442   of Solid Waste, Washington, DC.
443
444   US EPA (2007) (United States Environmental Protection Agency), Method 8015c, Nonhalogenated
445   organics by gas chromatography, in: Test Methods for Evaluating Solid Waste, Physical
446   Chemical Methods (SW–846), EPA, Office of Solid Waste, Washington, DC.
447
448   Walkley, A. & Black, C. A. (1934) .An examination of the Detjareff method for determining soil organic
449   matter, and a proposed modification of the chronic acid titration method. Soil Sci., 37, 29-38.
450   Walworth, J., Pond, A., Snape, I., Rayner, J., Ferguson, S., and Harvey, P. (2007). Nitrogen requirements
451   for maximizing petroleum bioremediation in a sub-Antarctic soil. Cold Regions Science and
452   Technology, 48, 84–91.
453
454   Yeung, . Y., Johnson, R. L., and Xu, J. G. (1997). Biodegradation of petroleum hydrocarbons in soil as
455   affected by heating and forced aeration. Journal of Environmental Quality, 26, 1511–1576.
456
457   Zajic, E., and Supplission, B. (1972). Emulsification and degradation of "Banker C" fuel oil by
458   microorganisms. Biotechnology and Bioengineering, 14, 331–343.
459
460