

Biodegradation of hydrocarbons in soil by microbial consortium

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Abstract

The bioremediation of hydrocarbon in contaminated soils by mixed cultures of hydrocarbon-degrading bacteria was investigated. The mixtures or consortia of bacteria, denoted as Consortium 1 and Consortium 2 consisted of 3 and 6 bacterial strains, respectively. Bacterial strains used in this study were from the Center for Research in Enzymes and Microbiology (CREAM) collection of strains, at Universiti Putra Malaysia, and were isolated from hydrocarbon-contaminated soil samples by enrichments on either crude oil or individual hydrocarbons as the sole carbon source. The strains were selected based on the criteria that they were able to display good growth in crude oil, individual hydrocarbon compounds or both. Their ability to degrade hydrocarbon contamination in the environment was investigated using soil samples that were contaminated with diesel, crude oil or engine oil. Consortium 2, which consisted of 6 bacterial strains, was more efficient at removing the medium- and long-chain alkanes in the diesel-contaminated soil compared to Consortium 1. Further, Consortium 2 could effectively remove the medium- and long-chain alkanes in the engine oil such that the alkanes were undetectable after a 30-day incubation period. Consortium 2 consisted predominantly of *Bacillus* and *Pseudomonas* spp.

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1. Introduction

Biodegradation of complex hydrocarbon usually requires the cooperation of more than a single species. This is particularly true in pollutants that are made up of many different compounds such as crude oil or petroleum and complete mineralization to CO₂ and H₂O is desired. Individual microorganisms can metabolize only a limited range of hydrocarbon substrates, so assemblages of mixed populations with overall broad enzymatic capacities are required to bring the rate and extent of petroleum biodegradation further. Microbial populations that consist of strains that belong to various genera have been detected in petroleum-contaminated soil or water (Sorkhoh et al., 1995). This strongly suggests that each strain or genera have their roles in the hydrocarbon transformation processes.

Further evidence for the cooperation of mixed cultures in biodegradation is apparent when Sorkhoh and co-workers (1995) observed a sequential change of the composition of the oil-degrading bacteria over a period of time in sand

samples that were contaminated with oil. Venkateswaran and Harayama (1995) reported similar observations in sequential enrichments in medium containing residual crude oil. In an earlier study using pure cultures, it was reported that after exhaustive growth of one strain on crude oil, the residual oil supported the growth of a second and third strain of bacteria (Horowitz et al., 1975).

Following the above findings, many studies of petroleum transformation have employed mixed bacterial or bacterial–fungal cultures in efforts to maximize biodegradation. Rambeloarisoa et al. (1984) demonstrated a consortium of 8 strains made up of members of 6 genera to be able to effectively degrade crude oil. Interestingly, only 5 of these strains were able to grow in pure cultures using a variety of hydrocarbons. However, when the other 3 strains were removed from the consortium, the effectiveness of the mixed culture was remarkably reduced. This further supports the theory that each member in a microbial community has significant roles and may need to depend on the presence of other species or strains to be able to survive when the source of energy is limited and confined to complex carbons.

The degradative capacity of any microbial consortium is not necessarily the result of merely adding together of

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the capacities of the individual strains forming the association. Many groups researching consortial biodegradation observed this. Komukai–Nakamura and co-workers (1996) reported the sequential degradation of Arabian light crude oil by two different genera. *Acinetobacter* sp. T4 biodegraded alkanes and other hydrocarbons producing the accumulation of metabolites. Following that, *Pseudomonas putida* PB4 began to grow on the metabolites and finally degrade aromatic compounds in the crude oil.

The advantages of employing mixed cultures as opposed to pure cultures in bioremediation have also been widely demonstrated. It could be attributed to the effects of synergistic interactions among members of the association. The mechanisms in which petroleum degraders benefit from synergistic interactions may be complex. It is possible that one species removes the toxic metabolites (that otherwise may hinder microbial activities) of the species preceding it. It is also possible that the second species are able to degrade compounds that the first are able to only partially (Alexander, 1999). Further research should be directed towards understanding the roles of individual members in influencing the effectiveness of a microbial association.

The objective of this study is to develop a formulation that can be directly employed onto a contaminated area. An “instant” formulation would hopefully be able to be applied in an instant following an oil spill to minimize the long-term damages to the environment such as those brought about by spreading, adsorption into soil and prolonged contamination.

2. Materials and methods

2.1. Microorganisms and culture conditions

Bacterial strains used in this study were from the Center for Research in Enzymes and Microbiology (CREAM) (at Universiti Putra Malaysia) collection of strains isolated from hydrocarbon-contaminated soil samples by enrichments on either crude oil or individual hydrocarbons as the sole carbon source. Hydrocarbon-degrading bacteria were characterized to genus level based on colony morphology and pigmentation, Gram staining and biochemical tests and identified according to the Bergey's Manual.

The liquid basal medium (BM) was composed of (g/l) K_2HPO_4 , 0.5; NH_4Cl , 2.0; KNO_3 , 2.0; and $MgSO_4$, 0.2. Artificial Seawater (ASW) consisted of (g/l) NaCl, 23.4; KCl, 0.75, and $MgSO_4$, 7.0. Inorganic nitrogen and phosphorus salts were prepared and added separately to BM and ASW (Foght et al., 1990). Tapis crude oil was obtained from Petronas Refinery at Malacca, Malaysia, and filter sterilized prior to addition to soil.

In order to obtain a standard inoculum, individual bacteria were grown for 18 h on Tryptone Soy Broth (OXOID) at 37°C in an orbital shaker at 150 rpm. The cells were then harvested by centrifugation, rinsed three times in sterile saline before being resuspended in sterile BM to yield

an absorbance reading of 0.5 at 540 nm. When used as an inoculum at 10% (v/w) the resulting colony forming unit (CFU)/g soil was between 1.62×10^7 and 2.34×10^7 cfu/g.

2.2. Microbial consortia preparation

Three microbial consortia were formulated by mixing equal proportions of pure bacterial cultures that were isolated from hydrocarbon-contaminated soils. Consortium 1 consisted of *Pseudomonas aeruginosa* strains S4.1 and S5 3 and *Bacillus* sp. Strain S3.2. Consortium 2 consisted of Consortium 1 as well as *Bacillus* sp. strains 113i and O63 and *Micrococcus* sp. strain S.

2.3. Diesel-contaminated soil

A preliminary study of the capacity of Consortia 1 and 2 to degrade petroleum hydrocarbons was conducted using soil that was contaminated with diesel fuel. The source of the soil was a diesel-refueling area of agricultural vehicles in an oil palm plantation in Sungai Buloh, Selangor, Malaysia.

One set of study consists of 300 g unsterilized soil in glass containers and 30 ml of bacterial inoculum. Three sets of study (triplicates) were conducted for each consortium. Control experiments were conducted by adding 30 ml of sterile BM to the soil.

2.4. Used engine oil-contaminated soil

Consortium 2 was selected to study the degradation of used engine oil in soil. The contaminated soil was obtained from the garage area for agricultural vehicles in the same plantation.

The soil was divided into two batches and one batch was autoclaved at 121°C for 30 min twice (24 h apart) to remove the indigenous microbial population. The other batch was left in natura. No crude oil was further added into the soil. 10% (v/w) Consortium 2 bacterial inoculum was introduced into the soil. As controls one set of sterile soil and another of the un-autoclaved soil were left un-inoculated.

2.5. Extraction and analyses of residual oil

The hydrocarbons in the mixture were harvested by the method described by Surzhko et al. (1995). Analyses were done by GC (Hitachi G3000) fitted with a flame ionization detector and SPB1 dimethylpolysiloxane capillary column (30 × 0.32 mm) (Supelco). The injector and detector were maintained at 290°C and the oven temperature was programmed to rise from 60°C to 280°C at 8°C min⁻¹ increment and held at 280°C for 5 min. The initial holding time at 60°C was also 5 min. H₂ was kept at 60 lb in⁻², compressed air 90 lb in⁻² and He 80 lb in⁻². The peak area was integrated by an attached Hitachi integrator (Chromato-Integrator D2500). Quantification of the *n*-alkanes was made

by comparing the peak areas of samples with that of the internal standard 2-nonanone (Del'Arco and França, 2001).

2.6. Microbial monitoring

The study was conducted at room temperature and monitoring was performed on Days 0, 15, 30, 45, and 60. To monitor cell numbers and biodegradation, 10 g of soil was removed from each container at the set times and resuspended in 10 ml of sterile saline in sterile centrifuge tubes. The mixture was vigorously shaken on a vortex mixer for 5 min and then the soil particulates were allowed to settle for 1 min before 0.1 ml of fluids were sampled for CFU counts.

3. Results

Based on their capabilities to grow on crude oil and/or individual hydrocarbons as their sole carbon source, six bacterial isolates were used in the construction of the bacterial mixtures or consortia used in this study (Table 1). Each of these isolates was selected based on the criteria that they were able to display good growth in crude oil, individual hydrocarbon compounds or both. From the trend seen in Table 1, substrate specificity of *Bacillus* spp. appeared to be restricted to the monoaromatic hydrocarbon compounds whilst the *Pseudomonas* and *Micrococcus* strains were able to grow on a variety of hydrocarbon groups tested in this study.

3.1. Soil analysis

The characteristics of the soil are presented in Table 2.

3.2. Diesel contaminated soil

For the degradation patterns of aliphatic compounds of diesel in the soil, a comparison was made between the hydrocarbon compositions of the soil at the beginning of the study (0 days) and at 60 days (Fig. 1). A gas chromatographic analysis of the hydrocarbon extracted with hexane at the

beginning of the study detected aliphatic compounds with carbon numbers of only between 14 and 24. The addition of the microbial mixtures Consortia 1 and 2 resulted in enhanced degradation of the middle- and long-chain aliphatic compounds in the soil compared to the soil that was not supplemented with any microbial consortium. The most significant reduction was seen when the soil was seeded with Consortium 2. The highest reduction by Consortium 2 was seen with C₁₄ where 57% of the alkane was removed. Between 47.40% and 81.43% of aliphatic compounds remained after 60 days of incubation with the presence of Consortium 2. Between 74.5% and 90.51% of the aliphatic compounds remained in the soil when inoculated with Consortium 1. Overall, better reduction of hydrocarbons was seen with Consortium 2. Also, biodegradation of short- and middle-chain aliphatic compounds was more extensive compared to the long-chain hydrocarbons.

The soil sample, which was contaminated with diesel, contained 1.0×10^5 cfu/g of indigenous soil microorganisms when plated on nutrient agar (Fig. 2). The control soil sample was not amended with the addition of any microbial consortium to study the effects of the inorganic nutrients in the Basal medium on the natural microbiota of the soil. In the consortia studies, adding 30 ml of bacterial inoculum onto 300 g of soil resulted in total cell counts of between 1.62×10^7 and 2.58×10^7 cfu/g soil on the day of inoculation. However, the microbial numbers continued to decrease from the time of inoculation up to 120 days when the study was terminated. The cell counts remained lower than the inoculum size at every sampling time. For Consortium 1, the CFU counts decreased from 1.62×10^7 cfu/g soil to 4.96×10^6 and 2.65×10^6 after 15 and 30 days, respectively. It continued to decrease when sampled at 45 days and remained at between 1.73×10^6 and 2.19×10^6 cfu/g up to 120 days. For Consortium 2, the numbers decreased from 2.37×10^7 cfu/g soil on inoculation day to 4.47×10^6 cfu/g at 30 days. Then the counts gradually increased and remained between 9.21×10^6 and 1.23×10^7 cfu/g until 120 days. In all instances, the cell counts in the inoculated soil remained higher than in the soil without any microbial mixture added.

Table 1

Isolates used in the construction of bacterial consortia, identification and the substrates that support their growth

Isolate	Isolate identification	Substrate ^a							
		Crude oil	Benzene	Toluene	Ethylbenzene	<i>o</i> -xylene	<i>n</i> -tetradecane	Octanol	Decanol
S3.2	<i>Bacillus</i> sp.	+	+	+	+	+	–	–	–
S4.1	<i>Pseudomonas</i> sp.	+	–	+	+	+	+	+	+
S5	<i>Pseudomonas</i> sp.	+	–	+	+	+	+	+	+
063	<i>Bacillus</i> sp.	–	+	+	+	+	–	+	–
113i	<i>Bacillus</i> sp.	+	–	+	+	+	–	–	–
S	<i>Micrococcus</i> sp.	+	–	+	–	+	+	+	–

– indicates isolate could not use substrate as carbon source.

^a+ indicates isolate could use substrate as carbon source.

Table 2

Profile description of diesel-contaminated soil and engine-oil-contaminated soil

Physical appearance	Diesel-contaminated soil	Engine-oil-contaminated soil
	Fine sandy clay loam	Coarse sandy loam
Clay (%)	16	10
Silt (%)	12	20
Fine sand (%)	51	17
Coarse sand (%)	19	52
Gravel (%)	0	0
pH	6.38	5.47
Nitrogen (%)	0.13	0.10
Available P (ppm)	1.9	7.0
Total petroleum hydrocarbons (%)	1.37	1.53

The addition of 30 ml of sterile basal medium into the soil without any addition of microbial mixtures resulted in the growth of the indigenous microbial population. The cell counts continuously increased from 1.00×10^5 at the beginning of the study to a maximum count of 9.95×10^5 cfu/g at 60 days. From then onwards, the numbers gradually decreased to 5.10×10^4 cfu/g at 120 days.

3.3. Used-engine oil-contaminated soil

The biodegradation of used-engine oil was studied using Consortium 2 following better biodegradation extent in the earlier studies with the diesel-polluted soil. Analysis of the hydrocarbon extracts from the untreated soil at the beginning of the study detected aliphatic compounds C₁₄–C₂₂ (Fig. 3a). A level of background biodegradation was seen when the contaminated soil was supplemented with sterile basal medium (Fig. 3b). Between 67.63% and 98.48% of C₁₅–C₂₂ remained after 30 days and 64.69% and 79.06% remained after 60 days in the un-inoculated control soil (Fig. 4). The addition of Consortium 2 into the soil significantly reduced the amounts of hydrocarbons in the oil-contaminated soil (Fig. 3c). With the exception of the branched alkanes pristane and phytane, the aliphatic compounds were reduced to undetectable levels 30 days after the addition of Consortium 2. At the end of the 60-day study, it was seen that Consortium 2 had further biodegraded pristane and phytane to 47.44% and 19.34% of their original values respectively.

The naturally occurring microorganisms in the soil numbered at 2.73×10^6 cfu/g soil when enumerated on nutrient agar. The addition of Consortium 2 suspension in Basal medium resulted in 5.13×10^7 cfu/g soil at the beginning of the study. The microbial counts in the inoculated soil increased to 7.76×10^8 cfu/g soil at 15 days but decreased to 1.35×10^8 cfu/g at 30 days and was about 5.26×10^6 cfu/g at 60 days post-inoculation when the study was terminated.

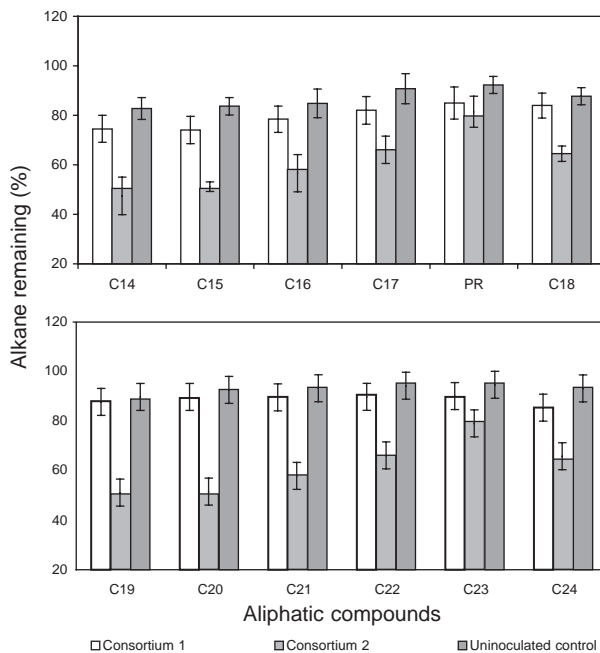


Fig. 1. Degradation of aliphatic compounds in diesel-polluted soil supplemented with Consortia 1 and 2 and without bacterial addition after 60 days of bioremediation process.

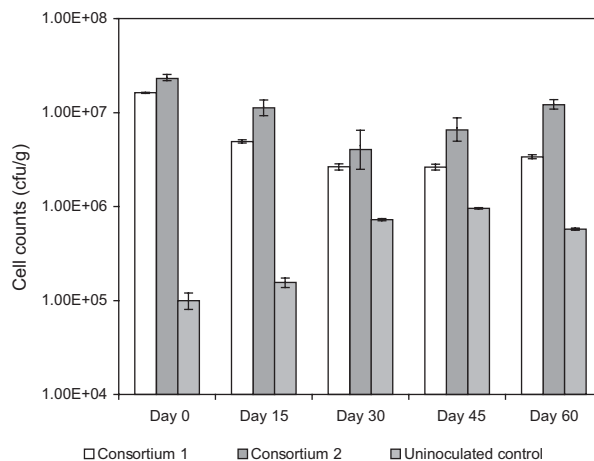


Fig. 2. Growth patterns in colony-forming units of bacteria in soil contaminated with diesel.

The addition of Basal medium to the un-inoculated soil did not enhance the growth of the indigenous microbial populations. There was a slight increase from 2.73×10^6 at 0 day to 3.63×10^6 cfu/g at 15 days. Their numbers remained low throughout the study (Fig. 5).

4. Discussion

The addition of basal medium to the diesel-polluted soil that was not inoculated with any microbial consortium

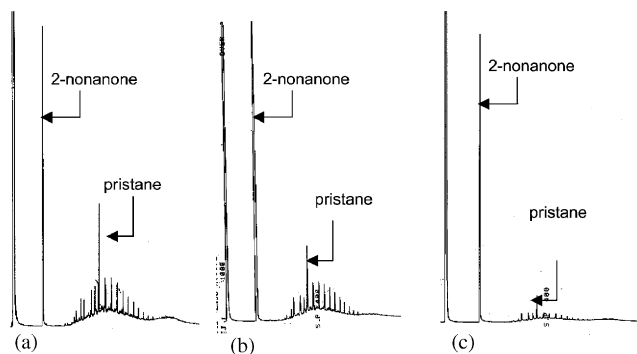


Fig. 3. (a) Chromatogram of hydrocarbons extracted from engine oil-polluted soil on day 0 (pre-inoculation). (b) Chromatogram of hydrocarbons extracted from engine oil-polluted soil on day 30 of control soil. (c) Chromatogram of hydrocarbons extracted from engine oil-polluted soil on day 30 of soil inoculated with Consortium 2.

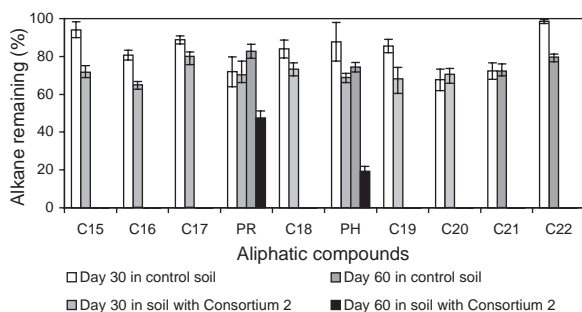


Fig. 4. Degradation of aliphatic compounds in engine oil-polluted soil by Consortium 2 and indigenous soil population after 60 days of bioremediation process. Aside from pristane and phytane, the aliphatic components of the engine oil could not be detected in the soil inoculated with Consortium 2.

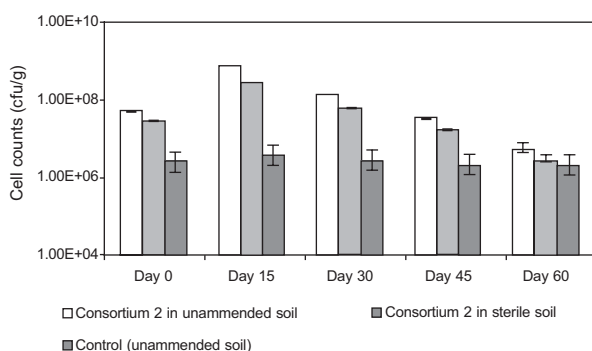


Fig. 5. Growth patterns in CFU of bacteria of Consortium 2 and indigenous bacteria in soil contaminated with engine oil.

resulted in an increase in the numbers of naturally occurring microorganisms in the soil. Their numbers increased by a tenfold until 45 days then gradually decreased again. This increase in numbers could be attributed to the addition of inorganic salts when basal medium was added to the soil. It is possible that due to the prolonged and level of

diesel contamination to the soil, the ideal ratios of inorganic to organic nutrients essential for microbial activities have been upset. Thomas et al. (1992) suggested that the ratio of C:N:P needs to be maintained at 120:10:1 to enable microbial growth and activity to occur. In the soil sample used in this study, the ratio of total petroleum hydrocarbons to nitrogen was 105:1. The nitrogen present in the soil was at least 10 times less than the levels that Thomas et al. (1992) recommended for microbial growth. At 1.9 ppm, the available phosphorus was in extremely low levels to support microbial proliferation with the high amounts of petroleum hydrocarbons present in the soil. The continuous spilling of diesel, due to carelessness of the operators, onto the ground at this refueling area had upset this ratio by way of increasing the ratios of carbon to both nitrogen and phosphorus.

Degradation of alkanes was more pronounced in the soil sample that was inoculated with Consortium 2. Consortium 2 consists of three *Bacillus* sp. strains, two *P. aeruginosa* strains, and one *Micrococcus* sp. strains whilst Consortium 1 consisted of one *Bacillus* strains and two *Pseudomonas* strains. It was proposed that *Bacillus* strains may play an important role for the more extensive biodegradation seen when Consortium 2 was applied to the soil. Investigations of polluted soil undergoing bioremediation is predominated by *Pseudomonas* spp. Very few papers have reported on the roles of *Bacillus* sp. in hydrocarbon bioremediation although there are several reports of bioremediation of pollutants by the action of *Bacillus* sp. occurring in extreme environments. Sorkhoh et al. (1993) isolated 368 isolates belonging to the genus *Bacillus* from desert samples. Two strains of *B. stearothersophilus* degraded 80–89% of crude oil (5 g/l) within 5 days at 60°C. In addition, Annweiler and co-workers (2000) described a *B. thermoleovorans* that degrades naphthalene at 60°C. More recently, Ijah and Antai (2003) reported *Bacillus* sp. being the predominant isolates of all the crude oil utilizing bacteria characterized from highly polluted soil samples (30% and 40% crude oil). When 5 soil isolates were compared, it was seen that one *Bacillus* sp. COU-28 was the best oil degrader compared to isolates belonging to *Micrococcus varians*, *P. aeruginosa*, *Vibrio* sp. and *Alcaligenes* sp. (Ijah and Antai, 2003). It was postulated that *Bacillus* species are more tolerant to high levels of hydrocarbons in soil due to their resistant endospores. There is thus growing evidence that isolates belonging to the *Bacillus* sp. could be effective in clearing oil spills.

Higher levels of hydrocarbon removal were seen with the medium chain alkanes compared to the longer chain alkanes. This is in agreement since short- and medium-chain alkanes are generally more easily degraded due to their lower hydrophobicity. Background degradation by the indigenous population of the contaminated soil also occurred, however, the extent of alkane removal by the microbial consortium was greater. Maximum reduction was seen with tetradecane where Consortium 2 removed 57% of the alkane. Compared to many reported soil studies, the level of reduction of hydrocarbons in this diesel-contaminated soil is relatively low.

Margesin and Schinner (1998) reported that between 10% and 30% of the initial soil pollution remains in soil after bioremediation techniques have been applied. Complete hydrocarbon reduction cannot occur due to their low bioavailability, especially as compounds are being used by the microorganisms as well as the accumulation of recalcitrant components.

Further, it is also possible that the soil component characteristic was detrimental against bacterial growth. The soil sample used in this study consisted mainly of clay particles, silt and fine sand (79% of the soil component). With fine soil particles, the pores between the sand grains are smaller and thus allow less room and path for water and gas flow. When liquid was added, the soil became waterlogged. It could thus be possible that the limited growth and degradation was due to the lack of oxygen in the soil. In a bioremediation study in the sand dunes of the British coast, it was seen that the decomposition process of buried oily beach sand appeared to cease when the sand became saturated with water, i.e. temporarily anaerobic. However, decomposition recommenced when the soil dried out (Rowland et al., 2000). Periodic tilling has commonly been employed in soil bioremediation studies to ensure an adequate supply of oxygen to the microorganisms (Del'Arco and França, 2001).

In contrast to the limited degradation of hydrocarbons in the soil that was contaminated with diesel, Consortium 2 was very capable of degrading the alkanes in the engine oil-contaminated soil. After 30 days of seeding with Consortium 2, C₁₅–C₂₂ was below detectable levels. 74.34% and 19.34% of pristane remained after 30 and 60 days of inoculation with Consortium 2, respectively. Compared to the other alkanes in the soil, the initial concentration of pristane was much higher. It could be possible that more time is required for the microbial populations to degrade this branched-alkane to below detection levels. Pristane has been reported to be a recalcitrant compound for attack by biodegrading microorganisms. Due to its resistance to biodegradation, it was commonly used as a chemical marker against which other biodegraded compounds are measured throughout a bioremediation study (Olivera et al., 2000; Wang et al., 1998). The observation that Consortium 2 could degrade compounds that were previously reported as very resistant to biodegradation promises potential that the bacterial mixture could be utilized in the bioremediation of a variety of hydrocarbon contaminants in soil. The biodegradation of pristane would also mean that the compound is not a suitable internal standard. Using the ratios of other hydrocarbon compounds against pristane may underestimate the degree of biodegradation due to the fact that the branched pristane are also biodegradable, even though at lower rates under most environmental conditions. However, the ratios of C₁₇/pristane can be used to differentiate between physical weathering and bioremediation (Wang et al., 1998). C₁₇ and pristane have about the same volatility and therefore weathering should be attributed for the loss of the compounds over time if their ratios remain the same.

On the other hand, if their ratios decrease over time, it would suggest that bioremediation is taking place, hence the higher rate of removal of C₁₇ compared to pristane.

Some removal of hydrocarbons was also seen in the soil which was not inoculated with Consortium 2. This removal could be attributed to the combined actions of indigenous microbial population of the polluted soil as well as abiotic weathering. Abiotic weathering processes in polluted soils include evaporation, photochemical oxidation, and adsorption onto particulate material. The indigenous microbial population of this particular soil sample numbered at approximately 10⁶ cfu/g soil. However, no tests were performed during this study to identify if these microorganisms were true hydrocarbon-degraders or if they were heterotrophic microorganisms that use other organic compounds as their carbon and energy sources. Furthermore, the addition of basal medium did not stimulate the indigenous bacterial population to multiply in numbers.

Further both appeared to require a period of time to adapt to the new niche. This could be due to several reasons. One possibility is that time is needed to adjust from growth in the rich Tryptone Soy Broth medium to growth on compounds that are less readily available such as hydrocarbons. Further, being hydrophobic compounds, hydrocarbons are poorly accessible to microbial cells. Heavily contaminated soils such as the sample used in this study contain a separate non-aqueous-phase liquid (NAPL) that may be present as droplets or film on soil surface (Stelmack et al., 1999). Many hydrocarbons are virtually insoluble in water and remain partitioned in the NAPL. The use of surfactants has been reported to increase the bioavailability of hydrophobic compounds in soil as well as in aqueous systems (Stelmack et al., 1999).

5. Conclusion

Consortium 2, a bacterial formulation consisting 6 strains isolated and purified from contaminated soil, could effectively degrade hydrocarbons in soil. However, the types of soil and hydrocarbon mixtures may determine the rate and extent of hydrocarbon remediation. Further studies could be done by evaluating the types and sizes of soil particles and how they interact with hydrocarbon compounds.

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