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Comparative Bioremediation of Petroleum Hydrocarbon-Contaminated Soil by Biostimulation, Bioaugmentation and Surfactant Addition

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Abstract: A bench-scale biopiling experiment was conducted to compare the ability of different techniques to enhance petroleum hydrocarbon bioremediation in a chronically contaminated soil. After 195 days, 10%-32% removal of TPHs (total petroleum hydrocarbons) occurred in unamended soil (control). Biostimulation by inorganic nutrient addition enhanced TPH removal (49%) confirming that bioremediation was nutrient limited and the soil contained a well-adapted hydrocarbonoclastic microbial community. The addition of organic amendments including green waste at 25% and 50% (w/w) and a commercial product called Daramend^{TM} had a further biostimulatory effect (50%-66%, 34%-59% and 69%-80% TPH removal respectively). Bioaugmentation using two commercially available petroleum hydrocarbon degrading microbial cultures with nutrients enhanced TPH removal in the case of RemActiv^{TM} (60%-69%), but had a marginal effect using Recycler 102 (49%-55%). The effect of a non-ionic surfactant in green waste amended soil was variable (52%-72% TPH reduction), but its potential to enhance biodegradation presumably by promoting contaminant bioavailability was demonstrated. High degradation of artificially added polycyclic aromatic hydrocarbons (PAHs) occurred after 106 days (75%-84%), but significant differences between the control and treatments were unapparent, suggesting that spiked soils do not reflect the behavior of contaminants in genuinely polluted and weathered soil.

Key words: Bioremediation, petroleum hydrocarbons, bioaugmentation, biostimulation, surfactant.

1. Introduction

The widespread and massive use of petroleum hydrocarbons over the past 200 years has left us with a legacy of contaminated sites which require remediation due to the unacceptable threat to human health and the environment [1]. In Australia, 30% to 40% of the estimated 80,000 contaminated sites are affected by hydrocarbon contaminants. Such contamination is commonly caused by leaking underground storage tanks and pipelines, oil refineries, land disposal facilities, wood treatment facilities and former manufactured gas plant sites [2]. Over time in the soil environment, petroleum hydrocarbons are subjected to a variety of natural processes including volatilization, biodegradation, photodecomposition, chemical oxidation, bioaccumulation, dispersion, diffusion, binding to soil and leaching to groundwater [3]. However, the time frame necessary for these processes to reduce hydrocarbon concentration is generally not feasible when viewed in the human context [4].

Traditionally, petroleum hydrocarbon contaminated soils have been dealt with by excavation and disposal to landfill. However, as landfills have become scarcer and more cost prohibitive, this method has become less feasible. Various physicochemical treatment techniques have been developed to clean up contaminated soil such as incineration, thermal desorption, chemical oxidation, immobilization and

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solvent extraction [5]. However, such treatments are generally expensive, energy intensive and not sustainable with respect to their environmental impacts which include damage to soil structure and toxicity issues associated with chemical additives [6]. Many of these techniques simply dilute or sequester the contaminants or transfer them from one environmental compartment to another and therefore do not eliminate the problem [3]. These limitations necessitate for a more economical and environmentally sound approach to remediate contaminated soils.

Microorganisms, namely heterotrophic bacteria and fungi have evolved a tremendous ability to metabolize simple and complex hydrocarbon contaminants [7]. By harnessing their metabolic ability, it is possible to remediate contaminated environments, a technique referred to as bioremediation [8]. This represents a valuable alternative to physicochemical remediation technologies as it enhances a natural process, resulting in the complete or partial biotransformation of organic contaminants into cell biomass and stable innocuous end products such as carbon dioxide and water [3, 5].

Petroleum hydrocarbons are biodegradable in geological time, although some in the human context can be particularly recalcitrant [4]. The slow rate of natural bioremediation is generally caused by a number of rate limiting factors including those imposed by the contaminant (biodegradability, bioavailability and concentration) and soil environmental factors (nutrients, oxygen, moisture, pH and temperature) which affect the growth and activity of microorganisms [8]. There are two main techniques to enhance soil bioremediation efficiency. Biostimulation refers to the adjustment of soil inorganic nutrients and/or organic nutrient substrates to stimulate the activity of contaminant degrading indigenous microorganisms. In cases where the natural microbial community cannot utilize the contaminants, bioaugmentation, the technique of introducing contaminant-degrading microorganisms can be employed [9]. Another developing bioremediation technique is the use of surface active agents (surfactants) to increase the bioavailability and therefore biodegradation of recalcitrant compounds [10]. These techniques are further discussed.

Petroleum hydrocarbons, the remnants of ancient plant and animal matter have entered the biosphere through seeps and erosion for millions of years [8]. As a consequence, metabolic pathways for their degradation have evolved and most soil ecosystems possess an adequate hydrocarbonoclastic microbial community capable of extensive hydrocarbon biodegradation [11]. Considering this, stimulating the native microbial community is a feasible option for bioremediation. Hydrocarbon contaminated soils, due to their high contaminant carbon concentration, generally contain limiting amounts of inorganic nutrients, particularly nitrogen and phosphorus which are major cellular components and therefore are essential for microbial growth and activity [6, 7]. This problem has been overcome by nutrient supplementation to restore the C:N:P ratio. Research has confirmed the effectiveness of biostimulation through nutrient addition [7, 12-14].

Organic amendments such as composts and manure have been investigated as potential co-substrates and environmentally friendly biostimulating agents for soil bioremediation [10]. These wastes themselves represent a pollution problem, but also a vast reservoir of nutrients and energy substrates which can stimulate vigorous growth of microbes [15]. Organic amendments are also beneficial to bioremediation as they improve soil properties such as soil structure and water holding capacity and generally possess a rich microbial population with hydrocarbon utilizing capabilities [16, 17]. When organic amendments are added to soil to enhance bioremediation, composting occurs whereby the organic amendments are degraded by microorganisms along with the contaminants, resulting in thermogenesis and the production of mature compost consisting of stable organic and inorganic compounds [1, 3]. Composting using animal manure and green waste has been shown to enhance hydrocarbon bioremediation [5, 16, 18]. The addition of compost, the mature end product of composting is also an effective biostimulation strategy [3]. For example, bioremediation of hydrocarbon contaminated soil has been enhanced using composted steer manure [19] and fish compost [20]. An important consideration when using organic amendments is their mix ratio in the soil, as an inappropriate ratio can retard or inhibit microbial activity [15, 18, 19].

Sometimes bioaugmentation, the technique of supplementing the soil with contaminant degrading strains, consortiums or enzyme preparations, may need to be employed to increase degradation rates of target contaminants [21]. This practice is usually adopted in the event of the deficiency or absence of indigenous contaminant degraders in the soil [22]. Some studies have concluded that bioaugmentation can enhance the bioremediation process [23-25], whereas others have observed no effect [2, 26]. The ubiquity of hydrocarbon degrading microorganisms makes the stimulation of native microbes more favorable over bioaugmentation, particularly in chronically contaminated soils where introducing contaminant degraders usually makes no difference because of their inability to compete with native microbes, predation and unsuitable soil conditions. Additional work is required for bioaugmentation to be a reliable and predictable adjuvant to bioremediation [22].

Limited contaminant bioavailability is often regarded as the key factor responsible for the persistence of many hydrocarbon contaminants in soil which should otherwise be biodegraded [6]. With increasing residence time in the soil, many hydrophobic organic contaminants become less bioavailable to microorganism due to interactions (adsorption and absorption) with soil constituents [27, 28]. The slow release of hydrophobic hydrocarbons from the solid soil phase to the aqueous phase where they are bioavailable leads to an impossibility in reaching the target of remediation [29]. Surfactant amended remediation has received significant attention to enhance bioremediation. Sorbed hydrocarbon contaminants in soil can partition into the hydrophobic cores of surfactant micelles, allowing them to be solubilized [30]. This promotes the mass transfer of unavailable hydrocarbons into the aqueous phase. Some studies have confirmed the beneficial effects of surfactants in bioremediation [22, 31, 32] whereas others have observed no effect [20, 33].

The aim of this study was to conduct a biotreatability experiment to compare the ability of the aforementioned approaches to enhance the bioremediation of petroleum hydrocarbons in a chronically contaminated, nutrient deficient soil. An ex-situ bioremediation technique called biopiling was employed at the bench-scale to establish soil treatments in order to evaluate the following research objectives:

(1) The effect of adjusting soil nutrients using inorganic fertilizers to stimulate the native soil microbial population and enhance bioremediation;

(2) The efficacy of conditioning nutrient adjusted soil with organic amendments (green waste and a commercial product) and the appropriate mix ratio to enhance bioremediation;

(3) The feasibility of adding commercially available contaminant degrading microbial cultures to nutrient adjusted soil to further enhance bioremediation;

(4) Whether the addition of a non-ionic synthetic surfactant in soil with nutrients and organic amendments can enhance the biodegradation rate by promoting contaminant bioavailability.

2. Materials and Methods

2.1 Soil Selection and Preparation

A sandy loam soil chronically contaminated with petroleum hydrocarbons was collected in composite samples from an old car service facility located in Newcastle, Australia. The soil was homogenised using a cement mixer to equally distribute the soil contaminants. To remove coarse fragments, the soil was passed through a 20 mm sieve. Preliminary analysis revealed high concentrations of TPHs (total petroleum hydrocarbons) (> 30,000 mg/kg) and no detectable levels of polycyclic aromatic hydrocarbons (PAHs). To investigate the bioremediation of the latter contaminant class, soil was artificially contaminated with creosote (Diggers Australia Pty Ltd.), a wood preservative consisting of PAHs (85%), phenolic compounds (10%) and *N*-, *S*- and *O*- heterocyclic compounds [2]. Half of the soil was spiked with creosote so that two different soils could be tested in this study: soil 1 (non-spiked, TPH contamination only) and soil 2 (spiked, TPH and PAH contamination). Creosote was added to achieve a PAH concentration of 1,000 mg/kg which was mixed through the soil by repeated tillage with a small spade.

2.2 Soil Treatments

The bench-scale bioremediation experiment was carried out from August 2010 to March 2011. To evaluate the research objectives, treatments were established for both soils 1 and 2. These included:

- Unamended soil (control);
- Soil with nutrients only (biostimulation);
- Soil with nutrients and 25% (w/w) green waste (biostimulation);
- Soil with nutrients and 50% (w/w) green waste (biostimulation);
- Soil with nutrients and a commercial organic amendment: Daramend™ (biostimulation);
- Soil with nutrients and a commercial bioaugmentation product: Recycler 102 (biostimulation + bioaugmentation);
- Soil with nutrients and a commercial bioaugmentation product: RemActiv™ (biostimulation + bioaugmentation);
- Soil with nutrients, 25% (w/w) green waste and a non-ionic synthetic surfactant (biostimulation + surfactant addition).

Sixteen biopiles were prepared in shallow rectangular containers, each receiving approximately 8.0 kg of soil. Small holes were drilled in the base of each container to allow for the excavation of excess water. Before the addition of soil, a small layer of gravel (2 cm) was placed on the base of each container with a geotextile cover to facilitate the drainage of biopiles and therefore prevent water logging.

Biostimulation was tested through the addition of nitrogen and phosphorus fertilizers. All biopiles except for the controls received liquid NH_4NO_3 (24.9% N, Paton Fertilizer Pty Ltd.) and crystals of water soluble $NH_4H_2PO_4$ (61% P, Paton Fertilizers Pty Ltd.). The nutrients were amended to attain a final proportion of carbon, nitrogen and phosphorus (C:N:P) of 100:5:1 by considering the initial carbon content of the hydrocarbon contaminants. Biostimulation was also tested through the addition of organic amendments. Partially composted green waste (primarily grass clippings) was obtained from composting bins and amended in both soils at two mix ratios: 25% (w/w) and 50% (w/w) (wet weight of soil). A commercial organic product called Daramend™ was also tested as a biostimulation agent. The chemical composition is given as: organic amendment (80%-90%), calcium carbonate (6%-8%) and soy lecithin (1%-5%) [34]. The addition rate of the product was determined by the supplier's recommendation including 1% (w/w) for soil 1 and 3.5% (w/w) for soil 2 considering the addition of PAH contaminants. The required mass of the organic amendments was spread on the soil surface of selected biopiles and incorporated throughout the entire matrix by repeated tilling with a small spade.

The feasibility of bioaugmentation was tested with two commercial microbial products, Recycler 102 (Allcrobe Pty Ltd.) and RemActiv™ (Ziltek Pty Ltd.). Recycler 102 is a liquid culture containing 27 different strains of free living microorganisms (gram + and gram-bacteria, actinomycetes and mycelia fungi) with capabilities to degrade aliphatic and aromatic hydrocarbons [35]. RemActiv™, also a liquid concentrate, contains selected microorganisms as well as a formulated nutrient mix [36]. The supplier's recommended doses were used including 4 mL/kg and 2 mL/kg for Recycler and RemActiv™ respectively.

One of two biopiles for each soil containing 25% (w/w) green waste was also amended with a non-ionic synthetic surfactant (TERIC® G9A6, Huntsman Corporation Pty Ltd.) at 1% (w/w).

2.3 Physical and Chemical Properties of Soil

Initial soil nitrogen, phosphorus and organic matter content was analyzed by the Inorganics Department of the Australian Laboratory Services Pty Ltd. (ALS) following the standards of the APHA methods 4500-Norg-D and 4500-NO_3-F, 4500 P-B and F [37], and AS1289.4.1.1[38] respectively. Soil moisture content and WHC (water holding capacity) was determined gravimetrically after desiccation at 105 °C for 24 hours following method 2A1 [39] and method SOG 4 [40] respectively. Biopiles were watered on a weekly basis by the addition of distilled water to maintain moisture at approximately 40-60% of the WHC. Soil pH was tested following method 4A1 prescribed by Rayment and Higginson [39] and was maintained at neutral by the addition of agricultural lime (Sibelco Australia Ltd.). To maintain aerobic conditions, biopiles were tilled on a weekly basis.

2.4 Hydrocarbon Analyses of Soil

Bioremediation efficiency was assessed by the analysis of remaining hydrocarbons as a function of elapsed time. TPHs in both soil 1 and 2 were monitored for 195 days (0, 26, 54, 90 and 195) in composite samples collected in duplicate and analysed by the Environmental Organics Department of the Australian Laboratory Services (ALS) Pty Ltd. using the US EPA method 8015A [41]. Following solvent extraction, TPH was measured by capillary gas chromatography coupled with a flame ionisation detector (GC-FID) and quantified against alkane standards over the range C_{10}-C_{36}. The total PAH concentration in soil 2 was monitored for 106 days (0, 42 and 106) and analysed by ALS according to the procedures of the US EPA method 8270B [41]. Following solvent extraction, PAH concentration was measured by capillary GC/MS (gas chromatography-mass spectrometry) in SIM (selective ion mode).

2.5 Bacterial Analyses of Soil

HAB (heterotrophic aerobic bacteria) and HDB (hydrocarbon-degrading bacteria) were enumerated as biological indicators of bioremediation efficiency. Soil samples (1 g) were serially diluted 10-fold in sterilised double distilled deionised Milli-Q water (0.2 micron filtered). 1 mL aliquots of the appropriate dilution(s) for each treatment were spread evenly on a Petri dish containing appropriate agar. For HAB, the growth medium was R2A agar which was incubated for 24 hours at 37 °C as described by Khan and Anjaneyulu [42]. HDB were grown on mineral silica gel-oil medium containing 0.05% (w/w) motor oil as described by Walker and Colwell [43] and incubated at 26 °C for14 days. The number of visible colonies were counted and reported as colony forming units per gram of soil ($CFU \cdot g^{-1}$ soil). HAB were enumerated throughout the bioremediation study (days 0, 21, 42, 63, 84, 113, 153 and 195) to assess changes over time for each treatment and between treatments at specific sampling points. HDB were enumerated once at the end of the study (day 195) to get an indication of the differences between treatments.

2.6 Statistical Analyses

Statistical significance of the data was evaluated by a one way analysis of variance (ANOVA) using JMP® version 7.0. The means were compared by the Tukey HSD multiple comparison test. The significance of differences among mean values was determined at a 95% confidence level ($p < 0.05$).

3. Results

3.1 Physicochemical Properties of Soil

Table 1 shows the initial physicochemical properties of the soil and green waste. The soil was characterized as a sandy loam with a neutral pH and deficient in nitrogen and phosphorus with respect to the high carbonaceous matter. Green waste proved to be an excellent source of these macronutrients with a more optimum C:N:P ratio. The green waste had a higher WHC which enhanced the WHC of the soil.

Table 1 Physicochemical properties of the soil and green waste. Each value is a mean of two replicates with the associated standard deviation.

Parameter	Soil	Green waste
pH	6.7 ± 0.2	5.9 ± 0.3
Moisture content (%)	10 ± 0.5	48 ± 1.5
Water holding capacity (%)	28 ± 1.0	68 ± 1.5
Organic matter (%)	4.8 ± 0.1	46.1 ± 0.2
Total nitrogen (mg/kg)	140 ± 0.3	18500 ± 0.5
Total phosphorus (mg/kg)	53 ± 0.1	1640 ± 0.2
C:N ratio	100:0.3	100:4.0
C:P ratio	100:0.1	100:0.4

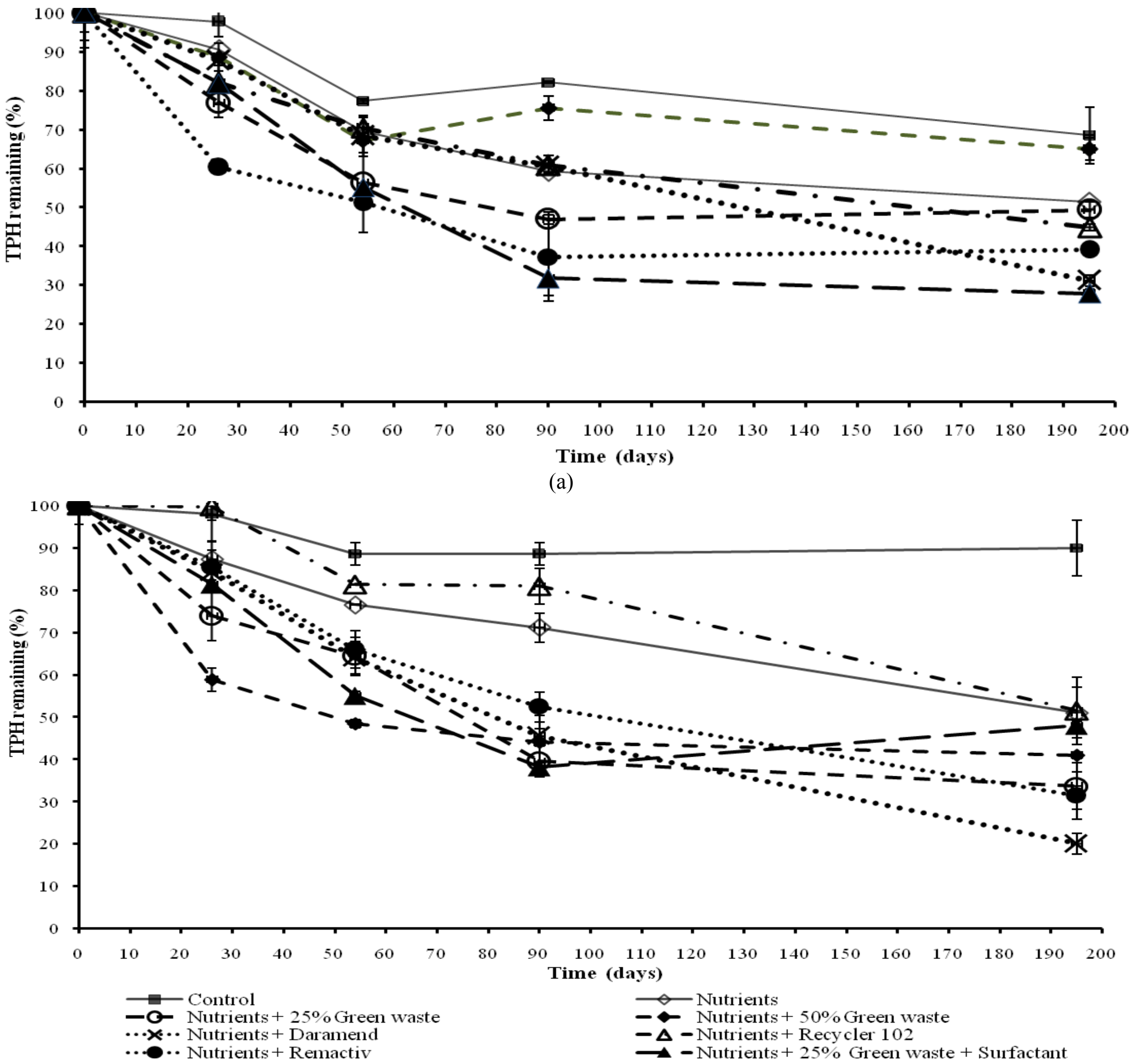
3.2 Chemical Assessment of Hydrocarbon Reduction

The effect of treatments on the reduction of TPH in soil 1 and 2 is shown in Figs. 1a and 1b respectively. Overall, a considerable decrease in TPH concentration occurred in most treated biopiles compared to the unamended controls (32% and 10% TPH reduction in soil 1 and 2 respectively) after 195 days of bioremediation. TPH reduction was significantly enhanced ($p < 0.05$) in the presence of nutrients alone (49% removal in both soils). Green waste addition had no significant effect ($p > 0.05$) in soil 1 (34% TPH reduction), compared to the control at 50% (w/w). However, in soil 2 this treatment was much more effective (59% TPH reduction) than the control ($p < 0.05$). In both soils, halving this amendment to 25% (w/w) resulted in further TPH reduction, but was variable between soils (50 and 66% in soil 1 and 2 respectively), with only soil 2 having a significant effect ($p < 0.05$) compared to nutrient addition alone. Daramend^{TM} had the greatest stimulatory effect on TPH removal in soil 2 (80%) and was the second best treatment in soil 1 (69%), both being significantly greater ($p < 0.05$) compared to treatments with nutrients and green waste.

Bioaugmentation using RemActiv^{TM} significantly enhanced ($p < 0.05$) TPH reduction with 60% and 69% removal in soil 1 and 2 respectively compared to nutrient and green waste amended biopiles and bioaugmentation with Recycler 102 in both soils. The latter treatment had no significant effect ($p > 0.05$) compared to nutrients only with 55% and 49% reduction occurring in soil 1 and 2 respectively. The effect of the non-ionic surfactant was highly variable, being the most effective treatment in soil 1 (72% TPH reduction), whereas it had no significant effect ($p > 0.05$) in soil 2 (52% TPH reduction).

The degradation of artificially added PAHs that occurred in soil 2 is recorded in Table 2. An overall high removal of ∑PAHs occurred during 106 days of bioremediation; however, the extent of degradation did not vary much between treatments and the control. After 106 days, amending soil with 50% (w/w) green waste followed by Daramend^{TM} proved to have the greatest effect on ∑PAH reduction (84% and 82% respectively) which were significantly higher ($p < 0.05$) compared to treatments with nutrients only (76%) and the surfactant/green waste (75%) but not compared to the control (79%) ($p > 0.05$). Both bioaugmented biopiles and the 25% (w/w) green waste biopile demonstrated a similar ∑PAH removal efficiency (80%) to the control during this time ($p > 0.05$).

The extent of degradation of PAH compounds appeared to be related to the number of aromatic rings (Table 2). Complete disappearance of naphthalene was observed in all treatments. PAH compounds with three aromatic rings also exhibited a high reduction. Higher molecular weight PAH compounds (≥ four aromatic rings) were more resistant to degradation. Reductions of five-ring compounds were lower in all



(a)

(b)

Fig. 1 Changes in TPH concentration during the bioremediation period in (a) soil 1 and (b) soil 2 biopiles, expressed as a percentage of the initial concentration. Data points represent the mean and standard variation of duplicate measurements.

biopiles containing organic amendments compared to the control ($p < 0.05$). This was also the case for six-ring compounds except with the presence of the surfactant. The diminution of ∑PAHs was accounted for mostly by the disappearance of low molecular weight PAHs, thus appearing to be extensive because these comprised more than 70% of the ∑PAH concentration. Overall, there was no clear pattern in the degradation of PAHs under the treatments tested, with the control having a similar or even greater effect compared to treatments.

3.3 Bacterial Counts

The number of colony forming HAB was assessed as biological indicators of bioremediation efficiency. Figs. 2a and 2b show that during the bioremediation period, the controls generally contained lower HAB counts compared to treated biopiles, suggesting a response of the soil microbial population to soil treatments. Nevertheless, control counts did increase by one to two orders of magnitude as the bioremediation period progressed. Green waste

Table 2 Percent removal of 16 US EPA PAH priority compounds in soil 2 biopiles after 106 days of bioremediation. Compounds are grouped according to number of aromatic rings. Reductions are expressed as a percentage of the initial concentration. Values represent the mean and standard deviation of two replicate measurements (nd = not detected).

PAH fraction	No. aromatic rings	Average composition in soil (%)	Control	Nutrients	Nutrients + 25% green waste	Nutrients + 50% green waste	Nutrients + Daramend$^{\text{TM}}$	Nutrients + Recycler 102	Nutrients + RemActiv$^{\text{TM}}$	Nutrients + 25% green waste + surfactant
Naphthalene	2	20-25	100	100	100	100	100	100	100	100
Acenaphthylene	3		100	100	100	100	100	100	100	100
Acenaphthene	3		80 ± 7	91 ± 6	100	100	100	90 ± 1	98 ± 1	100
Fluorene	3		98 ± 9	85 ± 2	100	100	100	87 ± 2	97 ± 1	100
Phenanthrene	3		99 ± 5	90 ± 4	100	100	100	93 ± 4	100	100
Anthracene	3		97 ± 4	79 ± 4	100	100	100	81 ± 1	100	100
Total	3	50	90 ± 7	88 ± 6	100*	100*	100*	89 ± 3	99 ± 1*	100*
Fluoranthene	4		31 ± 3	34 ± 2	66 ± 8	83 ± 23	57 ± 8	37 ± 2	29 ± 4	5 ± 2
Pyrene	4		28 ± 3	32 ± 2	0	16 ± 2	31 ± 3	38 ± 1	23 ± 4	2 ± 2
Benz[*a*]anthracene	4		40 ± 5	39 ± 4	33 ± 13	13 ± 7	26 ± 10	47 ± 2	31 ± 4	13 ± 1
Chrysene	4		39 ± 4	34 ± 3	24 ± 2	61 ± 1	58 ± 5	50 ± 1	21 ± 3	21 ± 1
Total	4	20-25	32 ± 4	33 ± 2	35 ± 4	42 ± 8*	45 ± 7*	39 ± 2	26 ± 4	7 ± 2$^{\bullet}$
Benzo[*b*]fluoranthene	5		47 ± 5	41 ± 5	8 ± 2	8 ± 3	2 ±2	10 ± 1	33 ± 4	7
Benzo[*k*]fluoranthene	5		42 ± 7	20	0	25 ± 2	0	73 ± 2	39 ± 9	20 ± 6
Benzo[*a*]pyrene	5		34 ± 11	12 ± 2	4	0	16 ± 2	29 ± 2	19 ± 4	13
Dibenz[*a*, *h*]anthracene	5		nd	nd	nd	nd	nd	nd	nd	nd
Total	5	< 4	40 ± 3	29 ± 3	8 ±1$^{\bullet}$	9 ± 2$^{\bullet}$	8 ± 3$^{\bullet}$	43 ± 2	29 ± 6	12 ± 2$^{\bullet}$
Benzo[*g.h.i*]perylene	6		47 ± 0	47 ± 0	25 ± 11	25 ± 0	32 ± 8	58 ± 8	38 ± 10	54 ± 0
Indeno[*1.2.3.cd*]pyrene	6		41 ± 7	33 ± 0	29 ± 8	39 ± 0	14 ± 6	53 ± 6	45 ± 8	58 ± 0
Total	6	< 1	44 ± 4	40 ± 0	30 ± 9$^{\bullet}$	34 ± 0$^{\bullet}$	23 ± 7$^{\bullet}$	55 ± 7	42 ± 9	57 ± 0
∑PAH			79 ± 4	76 ± 8	80 ± 6	84 ± 6	82 ± 3	80 ± 3	80 ± 4	75 ± 2

* Significantly higher compared to the control ($p < 0.05$);

$^{\bullet}$ Significantly lower compared to the control ($p < 0.05$).

amended biopiles consistently had significantly greater ($p < 0.05$) HAB counts compared to other treatments, by two to three orders of magnitude. Other treatments showed some important changes in HAB counts during the bioremediation period, however on average, these were not significantly higher ($p > 0.05$) compared to the control. Colony forming HDB were enumerated at the end of the bioremediation period to indicate differences between treatments. Similar to HAB, green waste biopiles exhibited the highest HDB counts being two orders of magnitude greater than the counts enumerated in other biopiles ($p < 0.05$). Although the nutrients only, bioaugmentation and Daramend$^{\text{TM}}$ treatments produced higher counts of HDB compared to the control, these were not significantly different ($p > 0.05$) (data not shown). In general, it was observed that bacterial counts did not reflect a response to the progress of hydrocarbon removal and the bioremediation efficiency.

4. Discussion

The efficacy of different bioremediation strategies in enhancing the biodegradation of petroleum hydrocarbon contaminants in soil was assessed in this study. In general, biostimulation, bioaugmentation and surfactant amended treatments enhanced TPH reduction and increased the soil bacterial population compared to the unamended controls. This suggests that the diminution in TPH concentration can be attributed to enhanced biodegradation. In contrast, ∑PAH removal in treatments was not significantly different to the control and therefore is not discussed extensively. The high ∑PAH removal (75%-84%) may be overly optimistic as the behaviour of spiked compounds is different to genuinely polluted soils. Caution is warranted when predicting the effect of bioremediation treatments from studies using spiked soils [3, 44].

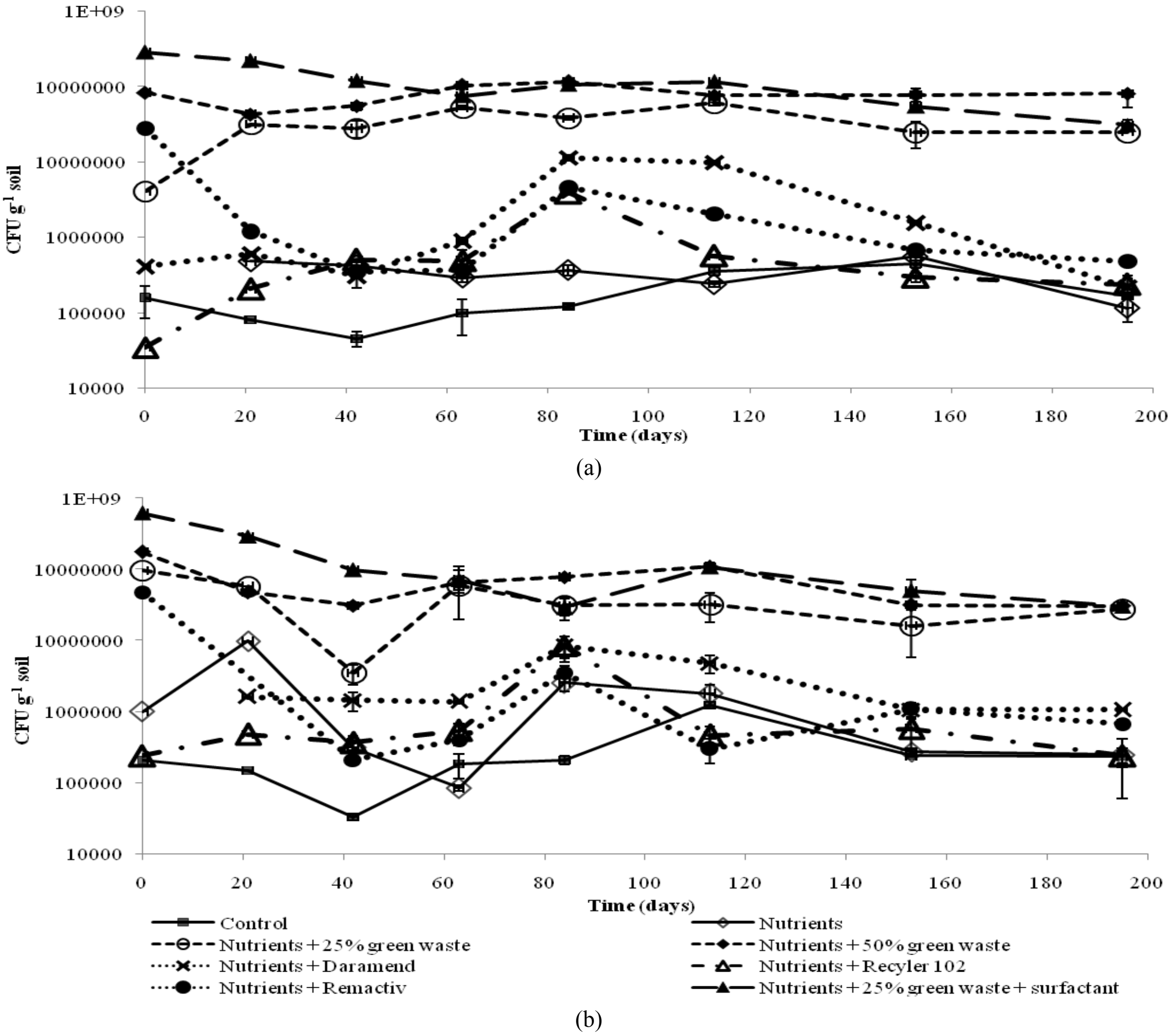


Fig. 2 Changes in the number of HAB during the bioremediation period (195 days) in (a) soil 1 and (b) soil 2 biopiles. Data points represent the mean and standard deviation of two replicates.

The complete disappearance of naphthalene and complete or near complete removal of three-ring PAHs was not surprising, as these PAHs can act as sole carbon and energy sources and their degradation is widespread among aerobic bacteria. Physical properties including low molecular weight, high volatility and slight water solubility may have accounted for their loss via abiotic processes. Larger PAHs (≥ four rings) were more resistant to degradation. This is attributed to their higher structural stability and reduced aqueous solubility. Biodegradation generally accounts for their removal, but at a slow rate as they cannot serve as sole carbon sources [43]. Limited metabolic ability to degrade these PAHs stems from biological factors are such as high activation energies and slow transport across the cell membrane as well as bioavailability issues [2].

4.1 Biostimulation with Inorganic Nutrient Addition

The addition of nitrogen and phosphorus fertilizers alone was effective in stimulating the indigenous microbial population, with 49% TPH reduction occurring in both soils compared to the unamended controls (10%-32%). This confirms that the indigenous microorganisms were physiologically adapted and capable of degrading hydrocarbons in this chronically contaminated soil, presumably due to prolonged contact with hydrocarbons which facilitates natural selection of diverse hydrocarbon degrading communities. It also suggests that one or both of these nutrients are limiting factors in natural bioremediation. Such findings are in agreement with previous studies [12-14].

The addition of nutrients needs to be evaluated on a site-specific basis. Indiscriminate reliance on nutrients to stimulate biodegradation may sometimes simply add to the expense of bioremediation [22]. The large quantity required in bioremediation projects is not environmentally sustainable considering that non-renewable resources are required to produce fertilizers. Ancillary effects of nutrient runoff are also an environmental concern. Nonetheless, biostimulation of native soil microbes by fertilizer addition remains the most common approach to enhance bioremediation [7].

4.2 Biostimulation Using Organic Amendments

Nutrient adjusted soil was conditioned with organic amendments including green waste and a commercial product (Daramend^{TM}) to evaluate their efficiency as biostimulating agents. The most notable effect of amending the soil with green waste was an increase in the bacterial counts in the contaminated soil. Composted organic wastes are known to sustain a high microbial diversity suggesting they are an effective and inexpensive microbial inoculant as well as a nutrient source [15]. Despite higher bacterial numbers, hydrocarbon degradation was lower than expected in green waste amended biopiles. This indicates that the contaminant degraders were utilising other carbon sources and/or the population of microorganisms from the green waste may have represented enough competition to slow the buildup of hydrocarbon-degraders [12]. Amending soil with 50% (w/w) green waste stimulated bioremediation in soil 2 (59%) however appeared to have an inhibitory effect in soil 1 (34%). Halving the amount to 25% (w/w) proved to have a greater stimulatory effect on bioremediation in each soil, with up to 66% removal in soil 2 and 50% TPH removal in soil 1. Thomas et al. [15] state that an inappropriate soil to organic amendment ratio can retard or inhibit microbial activity and therefore determining the most effective ratio is critical. Namkoong et al. [17] found that excessive addition of sewage sludge or compost to diesel contaminated soil retarded the degradation rate of the target contaminants. Antizar-Ladislao et al. [18] found that increasing or reducing the ratio of soil to green waste from 0.8:1 in aged coal-tar contaminated soil resulted in lower removals of PAHs. Adding excessive amounts of organic amendments may repress contaminant degrading enzymes as other carbon substrates are preferentially degraded [15].

The stimulatory effect on bioremediation by the commercial product Daramend^{TM} was much greater compared to green waste with 80% and 69% TPH removal occurring at 3.5% and 1% (w/w) respectively. The potential of Daramend^{TM} to extensively remove TPHs was apparent as no degradation plateau was observed. Previous pilot scale biopile studies have demonstrated the effectiveness of Daramend^{TM}, showing up to 84% removal of TPH at 1% (w/w) over a period of nine weeks [45]. This product is promoted due to its ability to enhance soil WHC and provide hydrophilic surfaces to enhance microbial growth [46].

Green waste and Daramend^{TM} amended treatments exhibited significantly lower reductions of five-ring and six-ring PAHs. Organically enriching soil has more frequently enhanced removal of high molecular weight PAHs. Wischmann and Steinhart [47] found that compost addition enhanced elimination of larger PAHs with 70% removal of benzo[*a*]pyrene. In pilot-scale testing, Seech et al. [46] applied Daramend^{TM} at 3% (w/w) to chronically contaminated soil and observed up to 71% and 60% removal of five- and six-ring PAHs respectively after 10 months of treatment. The recalcitrant nature of heavier PAHs in this study in the presence of organic amendments could be due to their sequestration into the organic matrix as concluded in studies by Wild and Jones [44] and Silva et al. [27]. In terms of TPH removal, the larger quantity of green waste (50% w/w) may have increased sorption and decreased bioavailability of contaminants. Whilst sorption processes achieve a reduction in overall risk to human health and the environment, the long term stability of pollutants that have been "locked up" (immobilised) in soil is uncertain [3].

Despite these limitations, organic amendments can be beneficial in bioremediation by reducing toxicity and ameliorating the physicochemical properties of soil. Directing organic wastes to bioremediation projects reduces demands for landfill and associated greenhouse gas emissions. However, a major concern is that mixing non-contaminated material with contaminated soil results in a far greater quantity of contaminated material to treat if the attempted remediation proves to be unsuccessful [3].

4.3 Bioaugmentation

Despite the presence of a native hydrocarbon-degrading community in the soil, the supplementation of nutrient adjusted soil with the exogenous microbial strains in RemActiv^{TM} significantly enhanced the extent of bioremediation compared to soil with nutrient amendment alone with up to 69% TPH removal. Inoculating nutrient adjusted soil with foreign contaminant degraders in Recycler 102 had no significant effect compared to nutrients only. It is common to find variable effectiveness of bioaugmentation using commercial cultures. Aldrett et al. [48] tested 12 commercial bioaugmentation products in crude oil contaminated soil in a laboratory scale experiment. After 28 days, only four products enhanced alkane and aromatic hydrocarbon biodegradation compared to a nutrient control. Jorgensen et al. [26] found that the addition of two commercial cultures (Oilbac and PRC 107 DTX) had no particular effect on the degradation of diesel and lubrication oil in soil undergoing composting with bark chips compared to nutrient addition alone. The non-effect of commercial cultures is usually attributed to chronically contaminated soils already possessing physiologically adapted native microorganisms with better soil distribution and metabolic capabilities to degrade hydrocarbons [12]. The strains in Recycler 102 may have been unable to compete favourably with the indigenous microbes and/or lacked the catabolic ability to attack the petroleum (TPH) compounds present in the soil as commercially cultured microorganisms are acclimated to degrade only certain contaminants [7].

Bioaugmentation is a controversial treatment concept and results are often contradictory [23]. Soil physicochemical conditions can be restrictive and its complex microbial ecology can limit the ability of exogenous strains to become self-perpetuating and carry out their specialised functions for an extended period [8]. Inoculation of soil with indigenous microbes directly isolated and cultured from the same soil has generally delivered better results [49] and should be the focus of future bioaugmentation attempts, since the strains are already adapted to their environment. The choice for bioaugmentation needs to be considered with the aid of sufficient laboratory and field pilot data on site specific degradation rates and survivability of added microorganisms.

4.4 Surfactant Addition

Low contaminant bioavailability in highly weathered soils can be a serious limitation in bioremediation. Microorganisms may have the metabolic capability to mineralize contaminants and yet fail to do so because they are unavailable to the cell [8]. The addition of a non-ionic synthetic surfactant attempted to ameliorate this potential problem. The effect of a non-ionic surfactant in soil also amended with nutrients and 25% (w/w) green waste was variable, being the most effective treatment in soil 1 (72% TPH reduction) and was initially effective in soil 2, but subsequent degradation became minimal. The beneficial effect of the surfactant appeared to take action earlier in the bioremediation process as observed by Delille et al. [20]. The TPH degradation plateaus observed in this treatment towards the end of the study may be due to toxicity issues. At concentrations over 100 mg/kg, many surfactants can reduce microbial activity by disrupting cell membranes (by interacting with phospholipids), thereby altering membrane permeability [50]. Dispersed oil can be toxic by encouraging excessive microbial uptake [6]. Delille et al. [20] attributed the enhanced toxicity of diesel residues in biopiles to surfactant addition (Brij 700) which encouraged only partial biodegradation of higher molecular weight PAHs.

There are other possible explanations for the decreased TPH reduction including competition of the surfactant as a carbon source for the microbial population [6]. Deschenes et al. [51] found that the addition of a surfactant (sodium dodecyl sulphate) caused four-ring PAHs to decrease more slowly than in untreated soil which was attributed to the surfactant being used as a preferential substrate as it was readily mineralised. Surfactants may also impede biodegradation by inhibiting bacterial attachment to contaminants [50]. An increased bioavailability of substrates in the green waste may have encouraged substrate competition. Strong-Gunderson and Palumbo [52] found that the surfactant Tween 80 enhanced the bioavailability of toluene and naphthalene but at the same time increased the availability of recalcitrant natural organic matter for biodegradation. Sometimes surfactants themselves can become sorbed to solid soil matrices making them ineffective in the mass transfer of hydrophobic contaminants [29].

A recent study found that only in the presence of a non-ionic surfactant (Empilan KR6) could combined bioaugmentation and biostimulation soil treatments enhance the rate of crude oil degradation compared to natural attenuation (50% compared to < 35% in other treatments) [22]. Aside from synthetic surfactants, other possible means of increasing hydrophobic contaminant bioavailability have been explored such as biodiesel [53] and canola oil [28]. Attempts have been made to isolate natural biosurfactants produced by hydrocarbon-degrading microbes [8]. When utilising surfactants, the fate and transport of dispersed hydrocarbons needs to be considered as well as the toxicity of the surfactant and its breakdown products [8]. More research is necessary to make their application a standard tool in bioremediation.

5. Conclusions

This study demonstrated that biostimulation, bioaugmentation and surfactant addition can enhance the removal of petroleum hydrocarbons in the selected soil. However, laboratory studies can produce overly optimistic results under controlled conditions, making it difficult to predict the performance of bioremediation strategies in the field where there is less control of environmental parameters and increased soil heterogeneity. An upgrade to field scale trials is therefore warranted to corroborate with the conclusions drawn from this study prior to undertaking field scale ventures.

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