Environmental Pollution, Volume 159, Issue 2, February 2011, Pages 515-523

MINERALISATIONOF TARGET HYDROCARBONS IN THREE CONTAMINATED SOILS FROM FORMER REFINERY FACILITIES

Marcie G. Towell [1], Jessica Bellarby [2], Graeme I. Paton [2], Frédéric Coulon[3], Simon J. T Pollard [3], and Kirk T. Semple [1*]

[1] Lancaster Environment Centre, Lancaster University, Lancaster LA1 4YQ, UK.

[2]Institute of Biological and Environmental Sciences, University of Aberdeen, Aberdeen AB24 3UU, UK

[3] School of Applied Sciences, Sustainable Systems Department, Cranfield University, Cranfield

*Corresponding author: Phone no. +44 1524 594534; Fax no. +44 1524 593985; e-mail: k.semple@lancaster.ac.uk

Abstract

This study investigated the microbial degradation of ^{14}C-labelled hexadecane, octacosane, phenanthrene and pyrene and considered how degradation might be optimised in three genuinely hydrocarbon contaminated soils from former petroleum refinery sites. Hydrocarbon mineralisation by the indigenous microbial community was monitored over 23 d. Hydrocarbon mineralisation enhancement by nutrient amendment (biostimulation), hydrocarbon degrader addition (bioaugmentation) and combined nutrient and degrader amendment, was also explored. The ability of indigenous soil microflora to mineralise ^{14}C-target hydrocarbons was appreciable; ≥ 16% mineralised in all soils. Generally, addition of nutrients or degraders increased the rates and extents of mineralisation of ^{14}C-hydrocarbons. However, the addition of nutrients and degraders in combination had a negative effect upon ^{14}C-octacosane mineralisation and resulted in lower extents of mineralisation in the three soils. In general, the rates and extents of mineralisation will be dependent upon treatment type, nature of the contamination and adaptation of the ingenious microbial community.

Capsule: Bioremediation strategy, native hydrocarbon concentrations and prior exposure histories of the microbial community influence hydrocarbon degradation in soil.

Keywords: Mineralisation, hydrocarbons, catabolic activity, nutrient addition and degrader amendment

1. Introduction

Anthropogenic hydrocarbon contamination of soil is a global issue throughout the industrialised world (Macleod et al., 2001; Brassington et al., 2007). In England and Wales alone, 12% of all serious contamination incidents in 2007 were hydrocarbon related (Environment Agency, 2005). Soil acts as a repository for many hydrocarbons, which is a concern due to their adverse impact on human health and their environmental persistence (Jones et al., 1996; Semple et al., 2001). Consequently, UK legislation has been introduced enforcing that if risk is posed to human, water or ecological receptors, contaminated land must be remediated to a level suitable for use (Paton et al., 2005). Sustainable remediation has therefore become a desirable option for the treatment of hydrocarbon-contaminated soils (Semple et al., 2001).

Predominately, hydrocarbon soil contamination is associated with the accidental spillage and leakage of refined petroleum derived products (Pollard et al., 1994). Consequently, potentially toxic and persistent contaminants, such as polycyclic aromatic hydrocarbons (PAHs) and mid to long-chained aliphatic hydrocarbons, are often dispersed ubiquitously in the environment (Wild and Jones, 1995). Polycyclic aromatic and heavier aliphatic hydrocarbons, which have a stable recalcitrant molecular structure, exhibit high hydrophobicity and low aqueous solubility, are not readily removed from soil through leaching and voltalisation. In addition, as these hydrocarbons ‘weather’ in soil, their bioavailability, phase partitioning, toxicity and degradability change (Brassington et al., 2007).

The degradation of petroleum-derived hydrocarbons has been widely studied and it has been established that microbial degradation is a key removal pathway of

hydrocarbons from the soil matrix (Atlas, 1981; Leahy and Colwell, 1990; Cerniglia, 1992; Budzinski et al., 1998; Loser et al., 1999; Bogane et al., 2003). Primarily, contaminant bioavailability plays a pivotal role in hydrocarbon degradation in soil. Additional factors that influence the degradation process included soil pH, moisture and organic matter content and hydrocarbon aqueous solubility, octanol-water partitioning coefficient and structure (Leahy and Colwell, 1990; Ramírez et al., 2008). Effective biodegradation is dependent upon optimal biological (microbial functionality and biomass size), chemical (bioavailability and nutrients) and physical (water holding capacity) parameters.

It is acknowledged that with 'weathering', the hydrocarbon bioavailability changes. This impacts upon the relative toxicity of the hydrocarbons and their susceptibility to biodegradation, assessed for bioremediation purposes (Brassington et al., 2007; Ramírez, et al., 2008). Bioassays may be used to evaluate the potential of indigenous soil microbial populations to degrade representative petroleum hydrocarbons, the impact these hydrocarbons have upon microbial activity and provide information on the bioavailability of contaminants in soils (Chaineau et al., 1995; Reid et al., 2000; Stokes et al., 2005).Through the measurement of lag phases (time prior to mineralisation reaching 5 %), and maximum rates and extents of a ^{14}C-labelled target hydrocarbon, in laboratory based mineralisation assays, biodegradation performance and justifiable end points can be accurately assessed (Macleod et al., 2001).

The hypotheses for this study were (i) soils contaminated with high concentrations of weather petroleum hydrocarbons have active indigenous microbial degrader populations able to degrade aliphatic and aromatic hydrocarbons and (ii) the addition

of nutrients and/or hydrocarbon degraders will increase catabolic activity in these soils, and enhance hydrocarbon degradation. To address these hypotheses, the following aims were considered: (i) to describe the microbial degradation of ^{14}C-labelled hexadecane, octacosane, phenanthrene and pyrene; (ii) to consider how degradation might be optimised in three genuinely hydrocarbon contaminated soils from former petroleum refinery sites, and (iii) to investigate enhancing microbial mineralisation of the target hydrocarbons by increasing microbial activity through (a) nutrient amendment, (b) addition of hydrocarbon degraders and (c) combined nutrient and degrader amendment.

2. Materials and methods:

2.1 Materials

Non-labelled and ^{14}C-labelled aliphatic and aromatic hydrocarbons; [1-^{14}C] hexadecane-n (specific activity = 12.0 mCi mmol^{-1}), [14, 15-^{14}C] octacosane (specific activity = 20.5 mCi mmol^{-1}), [9-^{14}C] phenanthrene (specific activity = 55.7 mCi mmol^{-1}) and [4, 5, 9, 10-^{14}C] pyrene specific activity = 48.5 mCi mmol^{-1}), were all supplied by Sigma Aldrich Co, UK. Ultima Gold liquid scintillation cocktail was obtained from Canberra Packard, UK. Merck, UK supplied the NaOH. The K_2HPO_4, KH_2PO_4 and salicylic acid were obtained from Fisher Scientific, UK. BDH Laboratory Supplies, UK supplied the NH_4NO_3. The hydrocarbon degrading inoculum and Bushnell Hass was obtained from Remedios Limited, UK. Oxoid Ltd, UK supplied the plate count agar (Tryptone Glucose Yeast Agar), minimal agar and Ringer solution. Internal standards utilised in the TPH extractions; Nonadecane-d_{40}, Triacontane-d_{62}, Naphthalene d_8, Phenanthracene-d_{10}, Chrysene-d_{12} and Perylene d_{12}, were all purchased from Sigma Aldrich, UK.

126

127 *2.2 Soil preparation and characterisation*

128 Three different soils collected at a depth of 5 – 20 cm from former oil refinery
129 facilities were labelled A, B and C (to maintain owner anonymity). Soil A and C were
130 untreated, whilst soil B had been previously biopiled. The soils were air-dried for 24 h
131 to enable them to be sieved through 2 mm, in order to remove stones, plant material
132 and facilitate mixing. Prior to air drying the field moisture content was determined in
133 triplicate by oven drying at 105°C for 24 h. Soils were then stored at 4^{o}C in the dark
134 before use.

135

136 The standard physical and chemical properties of each soil were analysed in triplicate
137 (Table 1). Following particle size analysis, determined using 40 ± 0.1 g soil (dry wt)
138 as per the hydrometer method (Klute, 1986), and based upon the USDA texture
139 classification system, soils A and B were categorised as clay and soil C as sandy clay
140 loam. The organic matter content (LOI) of each soil was measured by combustion at
141 450°C in a furnace for 24 h, according to ASTM Method D297487. Soils A and B
142 had organic matter content of ≤ 15%, whilst soil C contained the highest amount of
143 organic matter (26.47 ± 1.45 %) and organic carbon (15.39 ± 0.84%; Table 1). All
144 soils were determined to be slightly acidic with a pH range of 6.1 to 6.6, measured
145 with a PHM 220 lab pH meter (Model 657R-00) in a 1:5 soil (dry wt) to liquid
146 suspension, using 0.01 M calcium chloride ($CaCl_2$) solution and then separately with
147 deionised water.

148

149 Determination of the total petroleum hydrocarbon (TPH) concentration in the soil was
150 performed by sequential ultrasonic solvent extraction as described by Risdon et al

(2008). Total hydrocarbon petroleum (TPH), aliphatic and aromatic fractions were identified and quantified by gas chromatography-mass spectrometry using a Perkin Elmer AutoSystem XL gas chromatograph coupled to a Turbomass Gold mass spectrometer operated at 70 eV in positive ion mode. The column used was a Restek fused silica capillary column (30 * 0.25 mm internal diameter) coated with RTX®-5MS (0.25 µm film thickness). Splitless injection with a sample volume of 1µl was applied. The oven temperature was increased from 60°C to 220°C at 20°C min^{-1} then to 310°C at 6ºC min^{-1} and held at this temperature for 15 min. The mass spectrometer was operated using the full scan mode (range *m/z* 50-500) for quantitative analysis of target alkanes and PAHs. For each compound, quantification was performed by integrating the peak at specific *m/z*. External multilevel calibrations were carried out for both oil fractions, quantification ranging from 0.5 to 2500 µg ml^{-1} and from 1 to 5 µg ml^{-1}, respectively. Internal standards for the alkanes were nonadecane-d_{40} and Triacontane-d_{62} and Napthalene d_8, Phenanthracene-d_{10}, Chrysene-d_{12} and Perylene d_{12}. For quality control, a 500 µg ml^{-1} diesel standard and mineral oil were analysed every 10 samples. In addition, duplicate reagent control and reference material were systematically used. The reagent control was treated in exactly the same manner as the samples but contained no soil. The reference material was an uncontaminated soil of known characteristics, and was spiked with a diesel and mineral oil standard at a concentration equivalent to 16000 mg kg^{-1}. High concentrations of TPH (≥1.8%) were measured in all soils (despite soil B having undergone an active remedial treatment). Soil C contained the largest fraction of total aromatic hydrocarbons, and soil B aliphatic hydrocarbons.

175 Available ammonium and nitrogen was determined by extraction with 1 M KCl
176 solution following the method by Stockdale and Rees (1994). Acetic acid-extractable
177 phosphorus was determined by shaking 0.5 ± 0.1 g soil with 40ml of 2.5% acetic acid.
178 All extracts were then filtered through Whatman 44 filter paper and analysed on a
179 flow injection analyzer (FIAstar). No significant levels of ammonium, nitrate and
180 phosphorus were detected in any of the three soils
181
182 Enumeration of colony forming units (CFUs g^{-1} soil) for total heterotrophic and
183 hydrocarbon degrading microbes were estimated using the spread plate technique on
184 plate count agar and minimal agar supplemented with 0.1% diesel or enriched with
185 four separate hydrocarbons (hexadecane, octacosane, phenanthrene and pyrene). Soil
186 was extracted in a 1:10 ratio with quarter strength Ringer's solution, and then 1 ml of
187 the extract serially diluted with Ringer's solution. The resultant solutions (0.1 ml)
188 were then spread evenly over agar plates and incubated at 25 ± 1°C, with plate counts
189 performed at 4, 7, 10 and 15 days. Soil B had the largest indigenous heterotrophic
190 microbial community, and number of diesel and PAH degraders. The greatest number
191 of aliphatic hydrocarbon degraders (hexadecane and octacosane) was in soil C (Table
192 1).
193
194 *2.3 Soil Spiking with Target Hydrocarbons*
195 Prior to spiking, soils A, B and C were rehydrated with deionised water to 70% soil
196 water holding capacity (32, 31 and 26 mg per 100 g soil dry wt, respectively).
197 Samples of each soil, (120 g wet wt) were then spiked with $^{12/14}$C-labelled
198 hydrocarbons: hexadecane, octacosane, phenanthrene or pyrene using acetone as the
199 carrier solvent to give a final ^{12}C-hydrocarbon concentration of 50 mg kg^{-1} (dry wt)

200 with a related ^{14}C- activity of approximately 83 Bq g $^{-1}$ soil (dry wt). Each soil-
201 contaminant mixture was then blended following the method developed by Doick et al
202 (2003). Controls consisting of rehydrated soil (40 g wet wt) only were produced as
203 analytical blanks.
204
205 *2.4 Mineralisation Assays*
206 Biodegradation of the four ^{14}C-labelled petroleum hydrocarbons in each of the three
207 soils was measured (in triplicate) through the evolution of $^{14}CO_2$ produced using the
208 method developed by this group (Semple et al., 2006). The mineralisation assay was
209 performed in a 'respirometer': a modified 250 ml Schott bottle into which 10 ± 0.1 g
210 (wet wt) soil along with 30 ml of mineral salts medium (MBS) was placed. Glass vials
211 (7 ml) containing 1 ml of 1 M NaOH were attached to the respirometer Teflon-lined
212 lids to trap the $^{14}CO_2$ formed during the mineralisation assay.
213
214 Non-amended respirometers were prepared as outlined above, with 10 ± 0.1 g
215 hydrocarbon spiked soil (wet wt) and 30 ml of autoclaved MBS solution. To
216 investigate techniques to optimise the biodegradation of the target hydrocarbons,
217 respirometers were also prepared as above but with the following treatments: nutrient
218 amendment, hydrocarbon degrader amendment and combined nutrient and degrader
219 amendment. The carbon content of the soils was calculated (measured TPH + 50mg
220 kg^{-1} ^{12}C-PAH added) to be 23.2 mg, 32mg and 20.2 mg g^{-1} (dry wt) soil for soils A, B
221 and C, respectively; and nutrients were added to respirometers, to give a C: N: P soil
222 ratio of 100:10:1, (Leys et al., 2004). Nitrogen was given in the form of a 0.2 M
223 ammonium nitrate solution (prepared using autoclaved deionised water), added to
224 respirometers at 0.007 ml, 0.009 ml and 0.006 ml g^{-1} soil (wet wt), for soils A, B and

C, respectively. Potassium was added to respirometers (0.007 ml, 0.010 ml and 0.007 ml g^{-1} soil wet wt, for soils A, B and C respectively) via a 1 M potassium phosphate buffer (pH 7) prepared using anhydrous potassium orthophosphate and anhydrous dipotassium orthophosphate.. The degrader amendment comprised of a commercial mixed hydrocarbon degrader inoculum (identification unknown) able to utilise hexadecane octacosane, phenanthrene and pyrene as a carbon source for growth. The inoculum was cultured in autoclaved 3.27g l^{-1} Bushnell-Haas medium (prepared using deionised water, supplemented with 1000 mg l^{-1} salicylic acid and 1 % ethanol) at 20 ± 1°C on an orbital shaker at 100 rpm. After 2 days incubation (stationary phase), the hydrocarbon degrader inoculum was added to the respirometric flasks (0.1ml) such that numbers of cells rose to 10^6 cells g^{-1} dry wt soil. In respirometers amended with nutrients and/or degraders, MBS was added at 30 ml – amendment volume.

Respirometers containing only rehydrated soil were also prepared as analytical blanks. The respirometers were placed on an orbital shaker at 100 rpm and incubated in the dark at 20 ± 1°C. At regular intervals (24 h) over an incubation period of 23 d the $^{14}CO_2$ traps were replaced and 5ml Ultima Gold scintillation cocktail added to the sampled vial. Following overnight storage to normalize the effects of chemi-luminescence, the samples were counted by liquid scintillation counting LSC (Tri-Carb 2250CA) and quantified using standard counting protocols and automatic quench correction (Macleod and Semple, 2002).

2.5 Statistical analysis

Statistical analysis of the results after blank correction was performed in SigmaStat for Windows (Version 2.03 SPSS). All graphs were presented using SigmaPlot 2000

for Windows (Version 6.10, SPSS). The respirometric data for the four treatments (non amended, nutrient amended, inoculum amended and nutrient/inoculum amended) for each soil were tested for significance by analysis of variance (ANOVA), using Tukey and/or Student t-tests to investigate significant differences between lag phases, mineralisation rates and overall mineralisation extents, between different soil treatments, where $P \leq 0.05$.

3. Results

3.1. Mineralisation of target hydrocarbons in soils.

Hydrocarbon mineralisation in the three soils followed a standard 3-stage mineralisation curve. Firstly, there was a lag phase where the indigenous microbial community adapted to their freshly amended hydrocarbon and mineralisation was minimal (below 5%). Following this, there were increases in the rates of mineralisation, as displayed by an 'exponential' phase; after which catabolic activity reached a plateau (Figures 1-3).

The indigenous microbial communities in the three soils were able to mineralise each of the ^{14}C-target hydrocarbons (Figures 1-3; Tables 2-4). Rapid and extensive mineralisation ($\geq$ 40%) and high catabolic activity was observed in each of the soils spiked with ^{14}C-phenanthrene (Figures 1-3). This initial high catabolic activity resulted in significantly shorter ($P \leq 0.05$) lag phases of 21.72 ± 0.28 h in soil A, 9.03 ± 0.68 h soil B and soil C 4.23 ± 0.15 h, when compared to other ^{14}C-hydrocarbons in the same soil. Furthermore, maximum rates of ^{14}C-phenanthrene mineralisation were statistically quicker ($P \leq 0.05$) in the three soils, than those of ^{14}C-hexadaecane, ^{14}C-octacosane and ^{14}C-pyrene (Tables 2-4). Conversely, there was no statistical

difference in the overall extents of mineralisation for ^{14}C-phenanthrene between soils. Although, differences in maximum ^{14}C-phenanthrene mineralisation rates were evident, with a significantly faster ($P \leq 0.05$) rate of 2.37 ± 0.08 % h^{-1} occurring in soil C, in comparison to 1.11 ± 0.04 % h^{-1} for soil A and 1.12 ± 0.09 % h^{-1} for soil B. Similar observations were also observed for ^{14}C-phenanthrene lag phases in each of the soils; it took less time for extents of mineralisation to reach 5% in soil C followed by soil B, with soil A exhibiting the longest ^{14}C-phenanthrene lag phase (Tables 2-4). The higher levels of phenanthrene degradation in soils B and C were also reflected by a significantly larger ($P \leq 0.05$) number of phenanthrene degraders present in both soils (B, 4.1 x 10^5 ± 1.3 x 10^5 CFU g^{-1}) and (C, 4.3 x 10^5 ± 2.9 x 10^5 CFU g^{-1}), compared to soil A (1.8 x 10^4 ± 2.9 x 10^3 CFU g^{-1}).

In general, the ingenious microbial communities in the three soils exhibited less ability to mineralise ^{14}C-pyrene, compared to other ^{14}C-hydrocarbon amendments (Figures 1-3). This reduced response to pyrene resulted in significantly higher ($P \leq 0.05$) lag phases in soils A, B and C, in comparison to other ^{14}C-hydrocarbon lag phases (Table 2-4). This response correlated with lower numbers of pyrene degraders, in relation to hexadecane, octacosane and phenanthrene degraders, being present in soils A and C (Table 1). Significant differences in lag phases, maximum rates and overall extents of mineralisation for ^{14}C-pyrene were also apparent between the three soils. In soil B there were greater levels of catabolic ability with regard to the mineralisation of ^{14}C-pyrene than in soils A and C; the overall extent of ^{14}C-pyrene mineralisation was 1.72 and 1.43 times higher than in soils A and C, respectively (Figures 1-3). This higher catabolic ability for pyrene also resulted in a significantly faster ($P \leq 0.05$) maximum mineralisation rate of 0.29 ± 0.01 % h^{-1}, compared to 0.10

± 0.00 % h^{-1} in soil A and 0.19 ± 0.00 % h^{-1} in soil C. Furthermore, significantly more ($P \leq 0.05$) pyrene degraders were determined to be present in soil B, than in A and C (Table 1).

High levels of ^{14}C-hexadecane mineralisation were apparent in soils A and C with overall extents of mineralisation reaching 36.94 ± 0.53% and 50.48 ± 2.00%, respectively. Between soils, hexadecane associated catabolic activity was higher in soil C, which resulted in a significantly shorter ($P \leq 0.05$) ^{14}C-hexadecane lag phase and significantly faster ($P \leq 0.05$) maximum mineralisation rate than measured for other soils (Tables 2-4). However, there was no significant difference ($P \geq 0.05$) between the number of hexadecane degraders in soils A and C(Table 1). Conversely, the microbial community in soil B demonstrated significantly lower ($P \leq 0.05$) levels of mineralisation towards this hydrocarbon, with only 20.00 ± 0.66% ^{14}C-hexadecane mineralised over 23 d. This represents a decrease of 1.84 and 2.52 times on overall extents of mineralisation for ^{14}C-hexadecane in soils A and C, respectively. There was also significantly less ($P \leq 0.05$) ^{14}C-hexadecane mineralised in soil B in comparison to the overall extents mineralised for other ^{14}C-hydrocarbons in this soil (Figure 2, Table 3). Lower levels of catabolic activity towards hexadecane in soil B further correlated with the statistically significantly small ($P \leq 0.05$) number of hexadecane CFUs of $1.3 \times 10^4 \pm 4.1 \times 10^4$ measured in this soil.

The indigenous microbial communities of all three soils exhibited high levels of degradation for octacosane, with rapid and extensive ^{14}C-octacosane mineralisation observed over 23 d (Figures 1-3). In soils A and C, the microbial communities exhibited greater ability to mineralise ^{14}C-octaosane than ^{14}C-hexadecane, ^{14}C-

phenanthrene and ^{14}C-pyrene; overall extents of mineralisation for ^{14}C-octaosane were higher than for other ^{14}C-hydrocarbons in the same soil (Table 2 and 4). Between soils, there was lower octacosane degradative activity in soil B (45.84 ± 0.68%), with overall extents of mineralisation for ^{14}C-octacosane significantly higher ($P \leq 0.05$) at 54.93 ± 1.30%, and 54.32 ± 0.25% in soils A and C, respectively. No significant difference ($P \geq 0.05$) in the overall extents of mineralisation was observed between soils A and C. ^{14}C-octacosane was mineralised at a faster maximum rate of 0.91 ± 0.01 % h^{-1} in soil C, compared to 0.54 ± 0.04 % h^{-1} for soil A and 0.76 ± 0.00 % h^{-1} for soil B. Furthermore, the ^{14}C-octacosane lag phase for soil C was significantly shorter ($P \leq 0.05$) by 122.34 h and 36.7 h compared to soils A and B, respectively (Tables 2-4). This high level of octacosane degradation was also reflected by the presence of a significantly larger ($P \leq 0.05$) culturable octacosane degrader population compared to the other soils; 3.23 and 1.64 times more CFUs g^{-1} than in A and B, respectively (Table 1).

3.2 Enhancement of ^{14}C-target hydrocarbon mineralisation in soils

With nutrient amendment the microbial communities in soils B and C exhibited greater ability to mineralise ^{14}C-phenanthrene and ^{14}C-pyrene (Figures 2 and 3). In comparison to non-amended soils (to which no N and P were added) overall extents of mineralisation were significantly higher ($P \leq 0.05$) in soil B by 13.55% and 30.52%, and soil C by 40.41% and 17.33% for ^{14}C- phenanthrene and ^{14}C-pyrene, respectively. Nutrient addition also significantly enhanced ($P \leq 0.05$) maximum rates of mineralisation for ^{14}C-pyrene in both these soils (Tables 3 and 4). For example, the maximum rate of mineralisation for ^{14}C-pyrene was 0.26 ± 0.02 % h^{-1}, following nutrient addition in soil C, and 0.19 ± 0.00 % h^{-1} for the non-amended control.

Furthermore, nutrient amendment to soil B resulted in the significantly highest ($P \leq 0.05$) overall extent of mineralisation for ^{14}C-pyrene (61.32%) and the shortest lag phase of 104.84 ± 0.65 h. However, nutrient treatment did not result in (i) an increase in maximum rates of mineralisation and (ii) decrease in lag phases for ^{14}C-phenanthrene in soils B and C (Tables 3 and 4)**.** This treatment also had no significant effect ($P \geq 0.05$) on catabolic activity and the overall extent of mineralisation for ^{14}C-phenanthrene or ^{14}C-pyrene in soil A (Figure 1).

Conversely, in nutrient treated soil A, overall extents of mineralisation for ^{14}C-hexadecane and ^{14}C-octacosane were 2.19 and 1.45 times higher, respectively, than overall extents measured for non-treated soils (Figure 1). This increase in catabolic activity was further reflected by a significant decrease (compared to non-amended soils) ($P \leq 0.05$) in lag phases to (Table 2). Furthermore, with nutrient amendment maximum rates of mineralisation for ^{14}C-hexadecane increased by 0.35 % h^{-1} in soil A (Table 2). The overall extent of mineralisation for ^{14}C-octacosane also increased by ≥ 40% following nutrient amendment to 81.17 ± 0.93% in soil C. This was not significantly different to the overall extent mineralised in soil A (Figures 1 and 3). In soil B, overall extents of mineralisation for ^{14}C-hexadecane were also significantly higher ($P \leq 0.05$) with nutrient amendment (27.65 ± 0.70%), compared to non-amended soil (20.00 ± 0.66%), but was notably less than extents mineralised in soils A and C (Figures 1-3).

Hydrocarbon degrader treatment also significantly increased ($P \leq 0.05$) catabolic ability and overall extents of mineralisation for ^{14}C-hexadecane and ^{14}C-pyrene in all soils (Figures 1-3). This was the only treatment to increase the extent of

mineralisation for ^{14}C-hexadecane in soil C (Table 4). In comparison to non-amended soils, extents of mineralisation for ^{14}C-hexadecane increased by 42% (A), 9% (B) and 23% (C); and by 4% (A), 26% (B) and 24% (C) for ^{14}C-pyrene. However, this treatment did not enhance maximum rates of mineralisation for ^{14}C-hexadecane or reduce the lag phases for ^{14}C-hexadecane in soils B and C (Tables 3 and 4). Conversely, increases in pyrene associated catabolic activity for all degrader - amended soils were reflected by faster maximum mineralisation rates and significantly shorter ($P \leq 0.05$) lag phases, in comparison to non-amended soils (Tables 2-4). Compared to other treatments, degrader amendments also yielded the most extensive ^{14}C-pyrene mineralisation over 23 d for soils A and C (Figures 1 and 3). However, in soils A and B extents of mineralisation for ^{14}C-hexadecane were not statistically different ($P \geq 0.05$) for degrader and nutrient treatments (Figures 1 and 2).

With bioaugmentation the percentage of ^{14}C-phenanthrene mineralised in soil A decreased from 43.68 ± 1.80% (non-amended soil) to 27.37 ± 0.72%. This reduction in catabolic activity resulted in a significant increase in ^{14}C-phenanthrene ($P \leq 0.05$) lag phase (Table 2). When compared to non-amendment and other treatments in soil A, there was also a significant decrease ($P \leq 0.05$) in the maximum ^{14}C- phenanthrene mineralisation rate of ≥ 0.25 % h^{-1}**.** Conversely, in soil C the overall extent of mineralisation for ^{14}C-phenanthrene was 29.35% higher following degrader treatment in comparison to non-amended soil (Table 4). The overall extent of mineralisation for ^{14}C-phenanthrene was also 2.55 and 1.61 times higher than measured for degrader treated soils A and B, respectively (Figures 1-3). Enhanced octacosane related catabolic activity, was also observed in soils A and C following degrader addition. Compared to non-amended systems, extents of mineralisation increased from 54.93 ±

400 1.30% to 64.18 ± 0.65% in soil A; and 54.32 ± 0.25% to 84.82 ± 1.31% in soil C.
401 Although, overall extents of mineralisation for ^{14}C-octacosane and lag phases were
402 similar for nutrient and degrader treatments in soil C (Table 4). In comparison to non-
403 amended soil, the overall extent and maximum rate of mineralisation for ^{14}C-
404 octacosane significantly decreased by ≥ 10% and 0.61 % h^{-1} following degrader
405 treatment in soil B (Figure 2, Table 3).
406
407 Following combined nutrient + degrader treatment, an increase in catabolic activity
408 and ability to mineralise ^{14}C-phenanthrene was observed in all soils. Significantly
409 higher ($P \leq 0.05$) extents of ^{14}C-phenanthrene were mineralised in soils A, B and C, in
410 comparison to non-amended, nutrient and degrader amended soils (Figures 1-3).
411 Furthermore, in relation to nutrient and degrader treatments, ^{14}C-phenanthrene was
412 mineralised at significantly faster maximum rates ($P \leq 0.05$) with shorter lag phases in
413 all soils treated with nutrient + degrader (Tables 2-4). Between soils, there was no
414 significant difference ($P \geq 0.05$) in extents of mineralisation for nutrient + degrader
415 treatments. Although, a faster rate of mineralisation for ^{14}C-phenanthrene of 1.22 ±
416 0.02 % h^{-1} was observed for soil C, compared to 1.22 ± 0.02 % h^{-1} for soil A and 0.93
417 ± 0.01 % h^{-1} for soil B. However, in respect to non-amended soils, nutrient + degrader
418 amendment did not reduce lag phases or increase maximum rates of mineralisation for
419 all soils (Tables 2-4). Nutrient + degrader treatment also increased the overall extent
420 of mineralisation for ^{14}C-pyrene to 41.10 ± 0.36% in soil B and 30.90 ± 0.19% in C.
421 When compared to nutrient and degrader treatments, extents of mineralisation were
422 significantly lower ($P \leq 0.05$) in both soils for this treatment (Figures 2 and 3).
423 Furthermore, nutrient + degrader treatment was observed to have a negative effect
424 upon octacosane related catabolic activity in all soils (Figures 1-3). Compared to non-

amended soils, extent of mineralisation for ^{14}C-octacosane was 6.59, 6.21 and 6.57 times lower in soils A, B and C, respectively (Tables 2-4). This reduction in catabolic activity was reflected by statistically significant increases ($P \leq 0.05$) in ^{14}C-octacosane lag phases to 305.26 ± 3.02 h, 273.65± 9.52 h and 338.13 ± 8.79 h for soils A, B and C, respectively. Nutrient + degrader treatment also did not cause increases in the rate or extent of mineralisation for ^{14}C-hexadecane in all soils (Figures 1-3). However, a significant decrease ($P \leq 0.05$) in ^{14}C-hexadecane lag phases by 148 h and 4 h was observed for soils A and C, respectively, compared to non-amended soils (Tables 2-4). In respect to non-amended soils, this treatment also increased the overall extent of mineralisation for ^{14}C-pyrene in soils B and C by ≥ 9% (Figures 2 and 3).

4. Discussion

4.1 Catabolic activity of the indigenous microflora in soils A, B and C.

Many studies have documented the biological remediation of petroleum hydrocarbon contaminated sites (Trindade et al., 2005; Mancera-López et al., 2008; Liu et al., 2009) and it is widely acknowledged that for bioremediation strategies to be successfully applied, the potential for hydrocarbon biodegradation needs to be accurately assessed (Head, 1998). In this present study, the microbial degradation of ^{14}C-hexadecane, ^{14}C-octacosane, ^{14}C-phenanthrene and ^{14}C-pyrene was assessed in three genuinely hydrocarbon contaminated soils from former refinery facilities, which were all contaminated with high concentrations of TPH (≥1.8 % w/w) and contained only trace concentrations of phosphorus and no ammonium or nitrate. Optimising the degradation of the ^{14}C-target hydrocarbons through increasing microbial activity by nutrient amendment (nitrogen and phosphorus addition), addition of hydrocarbon degraders and combined nutrient and degrader amendment was also investigated. The

450 results reveal that prior to treatments, the indigenous microbial communities in the
451 three soils had the capability to degrade the representative ^{14}C-petroleum
452 hydrocarbons, and significant microbial activity was observed in all of the
453 contaminated soils. This is unsurprising due to the contamination histories of the soils,
454 which would result in extended exposure of the indigenous microbial communities to
455 selective hydrocarbons, and was further reflected by the large number of aliphatic and
456 aromatic degraders in each of the soils (Table 1).
457
458 Exposure of microbial populations to hydrocarbons in contaminated environments has
459 been observed to result in a selective enrichment in the numbers of indigenous
460 degrading organisms (Spain et al., 1980; Leahy and Colwell, 1990; Song et al., 1990;
461 Atlas, 1995) Furthermore, pre-exposure of microbial communities to hydrocarbons
462 can effect degradation, with biodegradation rates influenced by contaminant
463 concentration and prior exposure history (Spain and VanVeld, 1983; Aeolin et al.,
464 1989; Leahy and Colwell, 1990). This may be linked to hydrocarbon concentrations in
465 each of the soils. For example Grosser et al (1995) found that the mineralisation of 8
466 different ^{14}C-PAHs was faster and more extensive in genuinely hydrocarbon
467 contaminated soils containing 8 to 15 times higher concentrations of total
468 hydrocarbons(% g^{-1} dry wt soil). In this current study, levels of indigenous microbial
469 activity and mineralisation of ^{14}C-pyrene were significantly higher ($P \leq 0.05$) in soil B,
470 which contained the highest concentrations (mg kg^{-1}) of pyrene and total TPH.
471 Furthermore, the largest diesel and pyrene degrader populations and their ratio in the
472 heterotrophic microbial community were enumerated in this soil. In contrast, more
473 extensive ^{14}C-hexadecane and octacosane mineralisation occurred in soil C, which in
474 terms of aliphatic hydrocarbon burden contained the largest proportion of

hexadecane/octacosane (31.06% of total aliphatic hydrocarbon burden) in comparison to soils A (26.27%) and B (21.42%), and the highest ratio of aliphatic hydrocarbon degraders to total heterotrophic microbial population numbers. Comparable findings were observed for ^{14}C-hexadecane mineralisation in a study by Caparello and LaRock (1975), who reported an increase in ^{14}C-hexadecane mineralisation with increasing hydrocarbon burden. It was concluded that hydrocarbon concentrations indicate catabolic ability of indigenous microbes and their oxidising potential towards hydrocarbons (Caparello and LaRock, 1975).

In terms of soil physicochemical parameters, no definitive relationship was apparent between ^{14}C-hydrocarbon mineralisation parameters (lag phases, rates and extents of mineralisation) in the control soils and soils pH, organic matter content and particle composition. SOM is known to be predominant in orchestrating contaminant sequestration, and reducing bioavailability. High levels of SOM were measured in soil C, which contained ≥ 11% more soil organic matter (SOM) than soil A and B. However, differences in hydrocarbon degradability (between soils) as a result of declining bioavailability through contaminant sorption is suggested not to be a key factor in this study as all ^{14}C-hydrocarbons were freshly spiked in to the soils (Hatzinger and Alexander, 1997; Xing and Pignatello, 1997; Nam et al., 1998). Furthermore, studies have reported that freshly added PAHs can desorb from soil faster than they are degraded (Calvillo and Alexander, 1996; Cornelissen et al., 1998).

4.2 The role of treatments on microbial activity and implications for hydrocarbon bioremediation.

Depletion of essential nutrients can limit hydrocarbon biodegradation, especially in soils with high organic carbon contents (Breedveld and Sparrevik, 2000; Gallego et al., 2001; Joo et al., 2001; Bento et al., 2005; Horel and Schiewer, 2009). For example, Horel and Schiewer (2009) demonstrated the addition of N and P releasing fertilisers increased respiration (by 76% and 119%) over a 17 week period in contaminated Alaskan soil samples. In this current study**,** nutrient treatment in the form of nitrogen and phosphorus (added to soils at a C:N:P ratio of 100:10:1)had a stimulatory effect on catabolic activity, as in all soils extents of mineralisation, overall, significantly increased for the target ^{14}C-hydrocarbons. However, this increase in catabolic activity generally did not result in a decrease in ^{14}C-hydrocarbon lag phases, suggesting the added nutrients were being assimilated for microbial growth (Bamforth and Singleton, 2005). In certain instances, the addition of nutrients had no significant effect on the overall extents of ^{14}C-hydrocarbon mineralisation, when compared to extents of mineralisation in non-amended soils to which no nitrogen and phosphorus were added. Several studies have reported similar observations (Lehtomaki and Niemela, 1975; Seklemova et al., 2001; Chaineau et al., 2005; Chaillan et al., 2006; Ramírez et al., 2008). This has been attributed to the heterogeneity of soils, nutrient availability, toxicity of nutrient intermediaries and the presence of nitrogen fixing bacteria (Bossert and Bartha, 1984; Mills and Frankenberger, 1994; Seklemova et al., 2001; Sarkar et al., 2005). In this study there was no definitive evidence as to which of the factor/s may have contributed to non-increase in mineralisation parameters, following nutrient addition.

There have been a number of findings on the use of commercially available microbial inocula to remediate hydrocarbon contaminated soil, with some studies reporting

increased degradation and others negative or no effect on hydrocarbon degradation (Jobsen et al., 1974; Goldstein et al., 1985; Capelli et al., 2001; Bento et al., 2005). For example, Bento et al (2005) investigated the effect bioaugmentation had on diesel degradation in soil samples from Long Beach and Hong Kong over a 12 week period. Optimum biodegradation performance for Long Beach soil was achieved following the addition of a pre-selected microbial degrading consortium; whereas, this treatment had no significant effect on diesel degradation in the Hong Kong soils. In this current study, bioaugmentation of the three soils with a mixed hydrocarbon degrader consortium enhanced hexadecane, phenanthrene and pyrene degradation. However, degrader treatment was deemed to be less successful than the nutrient only treatment, which generally exhibited greater ^{14}C-mineralisation. Furthermore, the use of both nutrients and degrader inoculum as a combined soil treatment resulted in a decrease in extent of mineralisation for ^{14}C-octacosane, which reduced to $\leq$ 10 % in all three soils. This reduction in octacosane related catabolic activity may be the result of competition between indigenous and introduced degrader populations in the soil (Goldstein et al., 1985).

5. Conclusion

This study shows that the indigenous microbial communities in genuinely hydrocarbon-contaminated soils have the potential to actively and extensively degrade target aliphatic and aromatic hydrocarbons. Furthermore, by enriching the microbial activity and number of degrading microbes in hydrocarbon-contaminated soils, through biostimulation and bioaugmentation techniques, hydrocarbon degradation can be enhanced. However, the rate and extent of degradation will be dependent upon treatment type, contaminant structure, native hydrocarbon concentrations and

549 microbial community. In this current study, as the indigenous microbial populations
550 of all three soils had an established and capable degrader population, the addition of
551 degraders in this instance was not an effective solution to enhance the degradation of
552 the ^{14}C-contaminants in the soils. Similar observations have been noted and has been
553 suggested the advantages of a bioaugmentation remediation approach rarely outweigh
554 the costs involved. However, the successful bioremediation of hydrocarbon-
555 contaminated soils is dependent upon many soil, contaminant and microbial
556 parameters and should therefore be assessed on an individual site basis.
557
558 **Acknowledgments**
559 This work was co-funded by the Engineering and Physical Sciences Research Council
560 (EPSRC) and National Grid. It was further supported by the LINK Bioremediation
561 programme (BIOREM_35), the Environment Agency of England and Wales (EA) and
562 the Biotechnology and Biological Sciences Research Council BBSRC (Grant
563 BB/B512432/1).
564
565 **References**
566 Aeolin, C.M., Bradley, D.C., Pfaender, F.K., 1989. Adaptation of aquifer microbial
567 communities to the biodegradation of xenobiotic compounds: Influence of substrate
568 concentration and preexposure. Environmental Toxicology and Chemistry 8, 75 - 86.
569 Atlas, R.M., 1981. Microbial degradation of petroleum hydrocarbons: An
570 environmental perspective. Microbial Reviews 45, 180-209.
571 Atlas, R.M., 1995. Bioremediation of petroleum pollutants. International
572 Biodeterioration and Biodegradation, 317-327.

573 Bamforth, S.M., Singleton, I., 2005. Bioremediation of polycyclic aromatic
574 hydrocarbons: current knowledge and future directions. Journal of Chemical
575 Technology and Biotechnology 80, 723-736.
576 Bento, F.M., Camargo, F.A.O., Okeke, B.C., Frankenberger, W.T., 2005.
577 Comparative bioremediation of soils contaminated with diesel oil by natural
578 attenuation, biostimulation and bioaugmentation. Bioresource Technology 96, 1049-
579 1055.
580 Bogan, B.W., Lahner, L.M., Sullivan, W.R., Paterek, J.R., 2003. Degradation of
581 straight-chain aliphatic and high-molecular-weight polycyclic aromatic hydrocarbons
582 by a strain of Mycobacterium austroafricanum. Journal of Applied Microbiology 94,
583 230-239.
584 Bossert, I., Bartha, R., 1984. The fate of petroleum in soil ecosystems., in: (ed),
585 I.R.M.A. (Ed.). Macmillan Publishing Co, New York, pp. 434-476.
586 Brassington, K.J., Hough, R.L., Paton, G.I., Semple, K.T., Risdon, G.C., Crossley, J.,
587 Hay, I., Askari, K., Pollard, S.J.T., 2007. Weathered hydrocarbon wastes: A risk
588 management primer. Critical Reviews in Environmental Science and Technology 37,
589 199-232.
590 Breedveld, G.D., Sparrevik, M., 2000. Nutrient limited biodegradation of PAHs in
591 various soil strata at a creosote contaminated site. Biodegradation 11, 391-399.
592 Budzinski, H., Raymond, N., Nadalig, T., Gilewicz, M., Garrigues, P., Bertrand, J.C.,
593 Caumette, P., 1998. Aerobic biodegradation of alkylated aromatic hydrocarbons by a
594 bacterial community. Organic Geochemistry 28, 337-348.
595 Calvillo, Y.M., Alexander, M., 1996. Mechanism of Microbial Utilisation of Biphenyl
596 Sorbed to Polyacrylic Beads. Applied Microbiology and Biotechnology 45, 383 - 390.

597 Caparello, D.M., LaRock, P.A., 1975. A radioisotope assay for the quantification of
598 hydrocarbon biodegradation potential in environmental samples. Microbiology and
599 Ecology 2, 28-42.
600 Capelli, S.M., Busalmen, J.P., Sanchez, S.R., 2001. Hydrocarbon bioremediation of a
601 mineral-base contaminated waste from crude oil extraction by indigenous bacteria.
602 International Biodeterioration & Biodegradation 47, 233-238.
603 Cerniglia, C.E., 1992. Biodegradation of polycyclic aromatic hydrocarbons.
604 Biodegradation 3, 351 - 368.
605 Chaillan, F., Chaineau, C.H., Point, V., Saliot, A., Oudot, J., 2006. Factors inhibiting
606 bioremediation of soil contaminated with weathered oils and drill cuttings.
607 Environmental Pollution 144, 255-265.
608 Chaineau, C.H., Morel, J.L., Oudot, J., 1995. Microbial-Degradation in Soil
609 Microcosms of Fuel-Oil Hydrocarbons from Drilling Cuttings. Environmental Science
610 & Technology 29, 1615-1621.
611 Chaineau, C.H., Rougeux, G., Yepremian, C., Oudot, J., 2005. Effects of nutrient
612 concentration on the biodegradation of crude oil and associated microbial populations
613 in the soil. Soil Biology & Biochemistry 37, 1490-1497.
614 Cornelissen, G., Rigterink, H., Ferdinandy, M.M.A., van Noort, P.C.M., 1998.
615 Rapidly Desorbing Fractions of PAHs in Contaminated Sediments as a Predictor of
616 the Extent of Bioremediation. Environmental Science and Technology 32, 966 - 970.
617 Doick, K.J., Lee, P.H., Semple, K.T., 2003. Assessment of spiking procedures for the
618 introduction of a phenanthrene-LNAPL mixture into field-wet soil. Environmental
619 Pollution 126, 399-406.
620 Doick, K.J., Semple, K.T., 2003. The effect of soil:water ratios on the mineralization
621 of phenanthrene:LNAPL mixtures in soil. FEMS Microbiology Letters 220, 29 - 33.

622 Environment Agency, 2005. The UK Approach for Evaluating Human Health Risks
623 from Petroleum Hydrocarbons in Soils., Science Report P5-080/TR3
624 Gallego, J.R., Loredo, J., Llamas, J.F., Vazquez, F., Sanchez, J., 2001. Bioremediation
625 of diesel-contaminated soils: evaluation of potential in situ techniques by study of
626 bacterial degradation. Biodegradation 12, 325-335.
627 Goldstein, R.M., LMallory, L.M., Alexander, M., 1985. Reasons for possible failure
628 of inculation to enhance biodegradation. Applied Environmental Microbiology 50,
629 977-983.
630 Grosser, R.J., Warshawsky, D., Vestal, J.R., 1995. Mineralization of Polycyclic and
631 N-Heterocyclic Aromatic-Compounds in Hydrocarbon-Contaminated Soils.
632 Environmental Toxicology and Chemistry 14, 375-382.
633 Hatzinger, P.B., Alexander, M., 1997. Biodegradation of organic compounds
634 sequestered in organic solids or in nanopores within silica particles. Environmental
635 Toxicology and Chemistry 16, 2215-2221.
636 Head, I.M., 1998. Bioremediation: towards a credible technology. Microbiology 144,
637 599 - 608.
638 Horel, A., Schiewer, S., 2009. Investigation of the physical and chemical parameters
639 affecting biodegradation of diesel and synthetic diesel fuel contaminating Alaskan
640 soils. Cold Regions Science and Technology 58, 113-119.
641 Jobsen, A., McLaughlin, M., Cook, F.D., Westlake, D.W.S., 1974. Effects of
642 amendments on the microbial utilization of oil applied to soil. Applied Microbiology
643 27, 166-177.
644 Jones, K.C., Alcock, R.E., Johnson, D.L., Northcott, G.L., Semple, K.T., Woolgar,
645 P.J., 1996. Organic Chemicals in Contaminated Land: Analysis, Significance and
646 Research Priorities. Land Contamination and Reclamation 3, 189 - 197.

647 Joo, C.S., Oh, Y.S., Chung, W.J., 2001. Evaluation of bioremediation effectiveness by
648 resolving rate-limiting parameters in diesel-contaminated soil,. Microbiology and
649 Biotechnology 11, 607-613.
650 Klute, A., 1986. Particle-Size Analysis, in: A, K. (Ed.), Methods of Soil Analysis,
651 Part 1, Physical and Mineralogical Methods,
652 . ASA, ASSA, Madison, USA, pp. pp 338-409.
653 Leahy, J.G., Colwell, R.R., 1990. Microbial degradation of hydrocarbons in the
654 environment. Microbiology Review 54, 305 - 315.
655 Lehtomaki, M., Niemela, S., 1975. Improving microbial degradation of oil in soil.
656 AMBIO 4, 126-129.
657 Leys, M.N., Bastiaens, L., Verstraete, D., Springael, D., 2004. Influence of the
658 carbon/nitrogen/phosphorus ratio on polycyclic aromatic hydrocarbon degradation by
659 Mycobacterium and Sphingomonas in soil. . Applied Microbiology and
660 Biotechnology 66, 726-736.
661 Liu, W., Luo, Y., Teng, Y., Li, Z., Christie, P., 2009. Prepared bed bioremediation of
662 oily sludge in an oilfield in northern China. Journal of Hazardous Materials 161, 479-
663 484.
664 Loser, C., Seidel, H., Hoffmann, P., Zehnsdorf, A., 1999. Bioavailability of
665 hydrocarbons during microbial remediation of a sandy soil. Applied Microbiology and
666 Biotechnology 51, 105-111.
667 Macleod, C.J.A., Morriss, A.W.J., Semple, K.T., 2001. The role of microorganisms in
668 ecological risk assessment of hydrophobic organic contaminants in soils. Advances in
669 Applied Microbiology 48, 172-212.
670 Macleod, C.J.A., Semple, K.T., 2002. The adaptation of two similar soils to pyrene
671 catabolism. Environmental Pollution 119, 357 - 364.

672 Mancera-López, M.E., Esparza-García, F., Chávez-Gómez, B., Rodríguez-Vázquez,
673 R., Saucedo-Castañeda, G., Barrera-Cortés, J., 2008. Bioremediation of an aged
674 hydrocarbon-contaminated soil by a combined system of biostimulation-
675 bioaugmentation with filamentous fungi. International Biodeterioration &
676 Biodegradation 61, 151-160.
677 Mills, S.A., Frankenberger, W.T., 1994. Evaluation of phosphorus sources promoting
678 bioremediation of diesel fuel in soil. Bulletin of Environmental Contamination and
679 Toxicology 53, 280-284.
680 Nam, K., Chung, N., Alexander, M., 1998. Relationship between organic matter
681 content of soil and the sequestration of phenanthrene. Environmental Science and
682 Technology 32, 3785-3788.
683 Paton, G.I., Killham, K., Weitz, H.J., Semple, K.T., 2005. Biological tools for the
684 assessment of contaminated land: applied soil ecotoxicology. Soil Use and
685 Management 21, 487-499.
686 Pollard, S.J.T., Hrudey, S.E., Fedorak, P.M., 1994. Bioremediation of Petroleum- and
687 Creosote-Contaminated Soils: a Review of Constraints. Waste Management and
688 Research. 12, 173-194.
689 Ramírez, M.E., Zapiéna, B., Zegarraa, H.G., Rojasc, N.G., Fernández, L.C., 2008.
690 Assessment of hydrocarbon biodegradability in clayed and previous weathered
691 polluted soils. International Biodeterioration & Biodegradation 63, 347-353.
692 Reid, B.J., Jones, K.C., Semple, K.T., 2000. Bioavailability of persistent organic
693 pollutants in soils and sediments - a perspective on mechanisms, consequences and
694 assessment. Environmental Pollution 108, 103-112.
695 Reid, B.J., MacLeod, C.J.A., Lee, P.H., Morriss, A.W.J., Stokes, J.D., Semple, K.T.,
696 2001. A Simple 14C-Respirometric Method for Assessing Microbial Catabolic

697 Potential and Contaminant Bioavailability. FEMS Microbiology Letters 196, 141 -
698 146.
699 Risdon, G.C., Pollard, S.J.T., Brassington, K.J., McEwan, J.N., Paton, G.I., Semple,
700 K.T., Coulon, F., 2008. Development of an analytical procedure for weathered
701 hydrocarbon contaminated soils within a UK risk-based framework. Analytical
702 Chemistry 80, 7090-7096.
703 Sarkar, D., Ferguson, M., Datta, R., Birnbaum, S., 2005. Bioremediation of petroleum
704 hydrocarbons in contaminated soils: Comparison of biosolids addition, carbon
705 supplementation, and monitored natural attenuation. Envionmental Pollution 63, 187-
706 195.
707 Seklemova, E., Pavlova, A., Kovacheva, K., 2001. Biostimulation-based
708 bioremediation of diesel fuel: field demonstration. Biodegradation 12, 311-316.
709 Semple, K.T., Dew, N.M., Doick, K.J., Rhodes, A.H., 2006. Can microbial
710 mineralization be used to estimate microbial availability of organic contaminants in
711 soil? Environmental Pollution 140, 164-172.
712 Semple, K.T., Reid, B.J., Fermor, T.R., 2001. Review of composting strategies to
713 treat organic pollutants in contaminated soils. Environmental Pollution 112, 269 -
714 283.
715 Song, H.-G., Wang, X., Bartha, R., 1990. Bioremediation potential of Terrestrial Fuel
716 Spills. Applied and Environmental Microbiology 56, 652-656.
717 Spain, J.C., Pritchard, P.H., Bourquin, A.W., 1980. Effects of adaptation on
718 biodegradation rates in sediment/water cores from estuarine and freshwater
719 environments. Applied and Environmental Microbiology 40, 726 - 734.
720 Spain, J.C., VanVeld, P.A., 1983. Adaptation of natural microbial communities to
721 degradation of xenobiotic compounds: Effects of concentration, exposure time,

722 inoculum and chemical structure. Applied and Environmental Microbiology 45, 428 -
723 435.
724 Stockdale, E.A., Rees, R.M., 1994. Relationships between Biomass Nitrogen and
725 Nitrogen Extracted by Other Nitrogen Availability Methods. Soil Biology &
726 Biochemistry 26, 1213-1220.
727 Stokes, J.D., Wilkinson, A., Reid, B.J., Jones, K.C., Semple, K.T., 2005. Prediction of
728 Polycyclic Aromatic Hydrocarbon Biodegradation in Contaminated Soils Using an
729 Aqueous Hydroxypropyl-β-Cyclodextrin Extraction Technique. Environmental
730 Toxicology and Chemistry 24, 1325-1330.
731 Trindade, P.V.O., Sobral, L.G., Rizzo, A.C.L., Leite, S.G.F., Soriano, A.U., 2005.
732 Bioremediation of a weathered and a recently oil-contaminated soils from Brazil: a
733 comparison study. Chemosphere 58, 515-522.
734 Wild, S.R., Jones, K.C., 1995. Polynuclear aromatic hydrocarbons in the United
735 Kingdom environment: a preliminary source inventory and budget. Environmental
736 Pollution 88, 99-108.
737 Xing, B., Pignatello, J.J., 1997. Dual-mode sorption of low-polarity compounds in
738 glassy poly(vinylchloride) and soil organic matter. Environmental Science and
739 Technology 31, 792-799.
740
741
742

743 **Figure legends**

744 **Figure 1.** Mineralisation of (A), ^{14}C-hexadecane (B) ^{14}C-octacosane (C) ^{14}C-
745 phenanthrene (D) ^{14}C-pyrene occurring over 23 days in soil A – an industrial surface
746 soil obtained from a disused oil refinery. The soil was amended with different
747 treatments, no treatment (●) nutrient treatment (■), hydrocarbon degrader treatment
748 (▲) and nutrient + degrader (○). Error bars where visible are 1 SEM based on n=3.

749

750 **Figure 2.** Mineralisation of (A), ^{14}C-hexadecane (B) ^{14}C-octacosane (C) ^{14}C-
751 phenanthrene (D) ^{14}C-pyrene occurring over 23 days in soil B – an industrial surface
752 soil remediated with biopiling. The soil was amended with different treatments: no
753 treatment (●), nutrient treatment (■), hydrocarbon degrader treatment (▲) and
754 nutrient + degrader treatment (○). Error bars where visible are 1 SEM based on n=3.

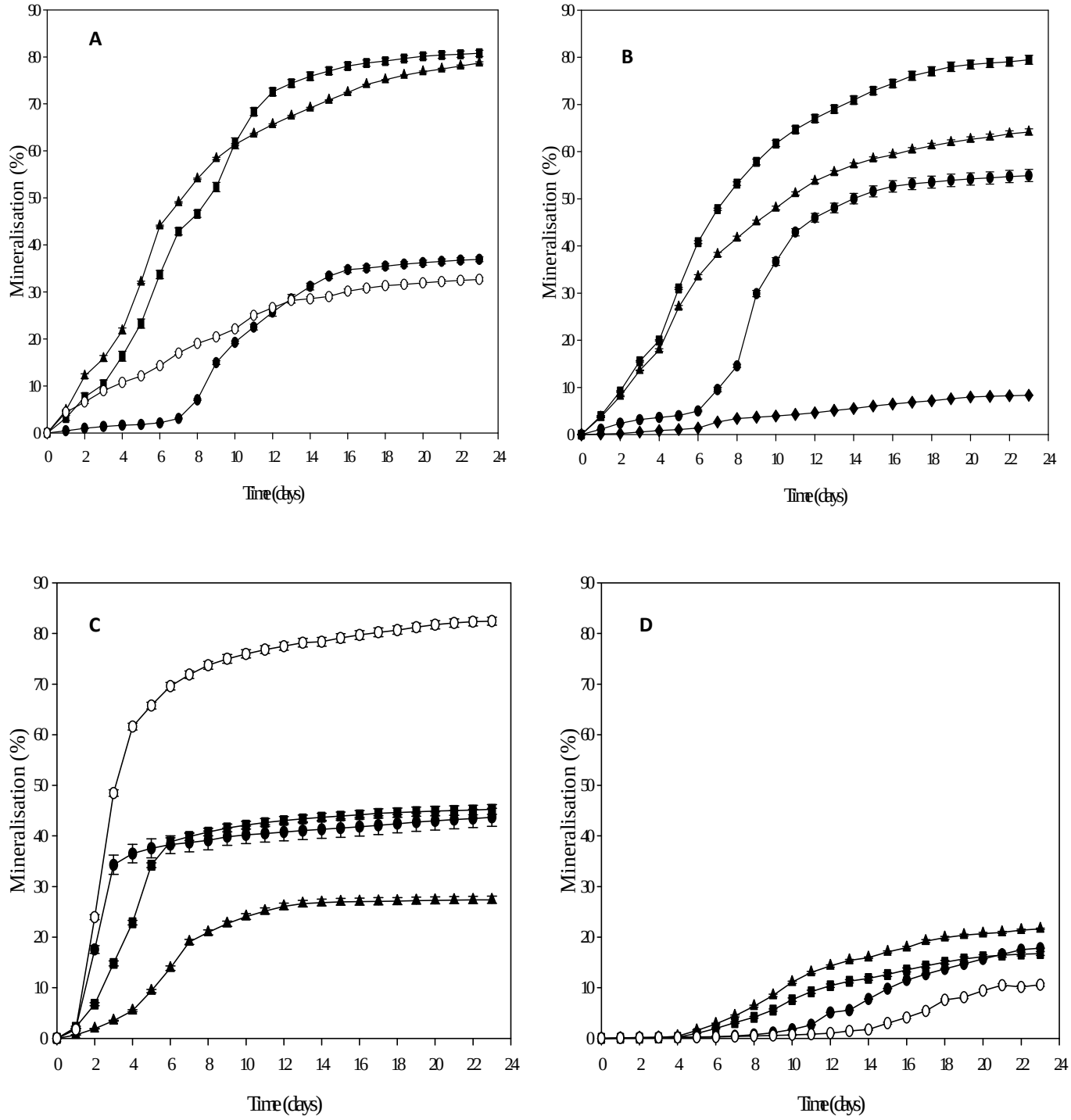
755

756 **Figure 3.** Mineralisation of (A), ^{14}C-hexadecane (B) ^{14}C-octacosane (C) ^{14}C-
757 phenanthrene (D) ^{14}C-pyrene occurring over 23 days in soil C – an industrial surface
758 soil from an old oil refinery site. The soil was amended with different treatments: no
759 treatment (●), nutrient treatment (■), hydrocarbon degrader treatment (▲) and
760 nutrient + degrader treatment (○). Error bars where visible are 1 SEM based on n=3.

761

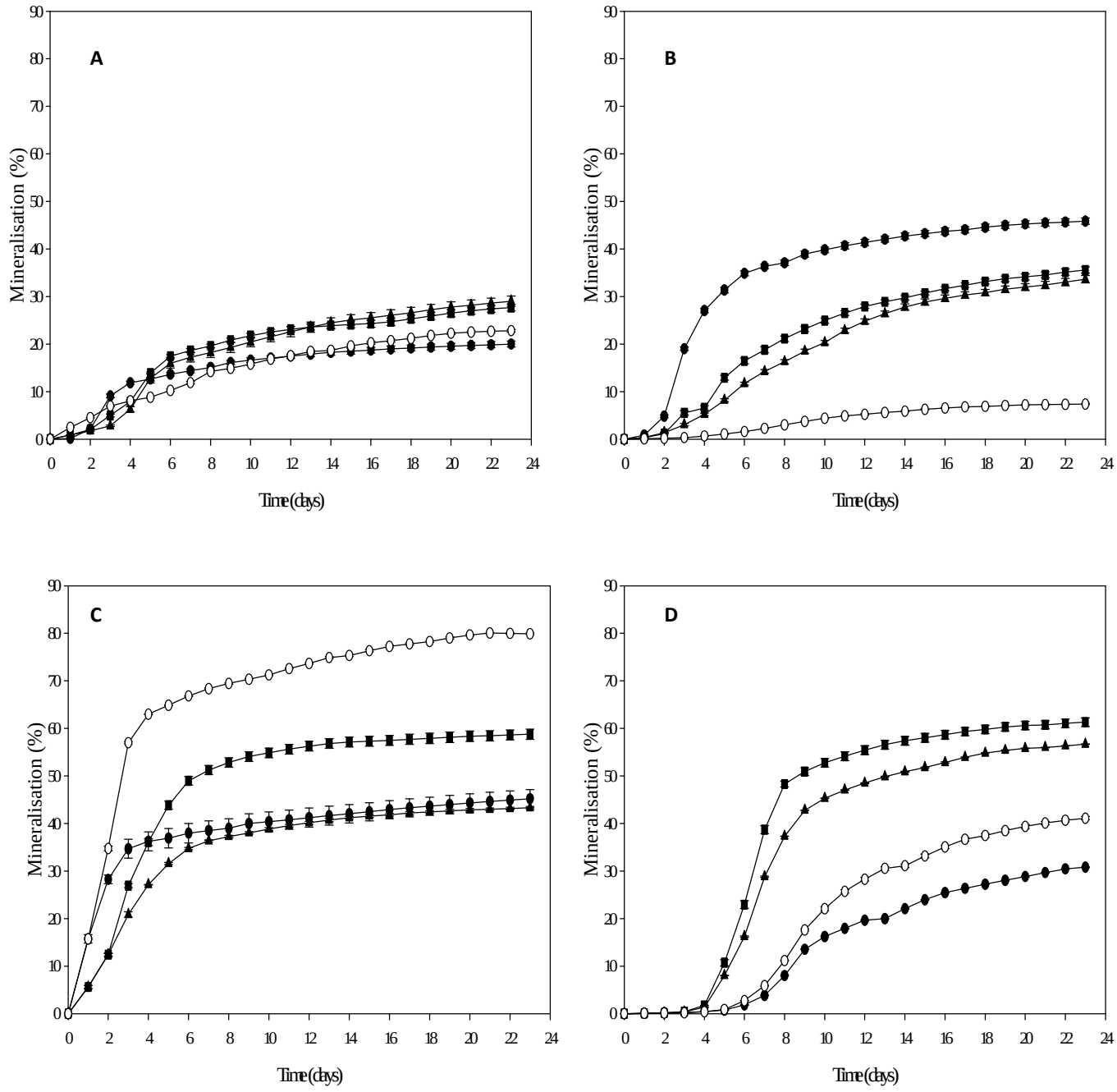
762

763 Figure 1



766 Figure 2

767

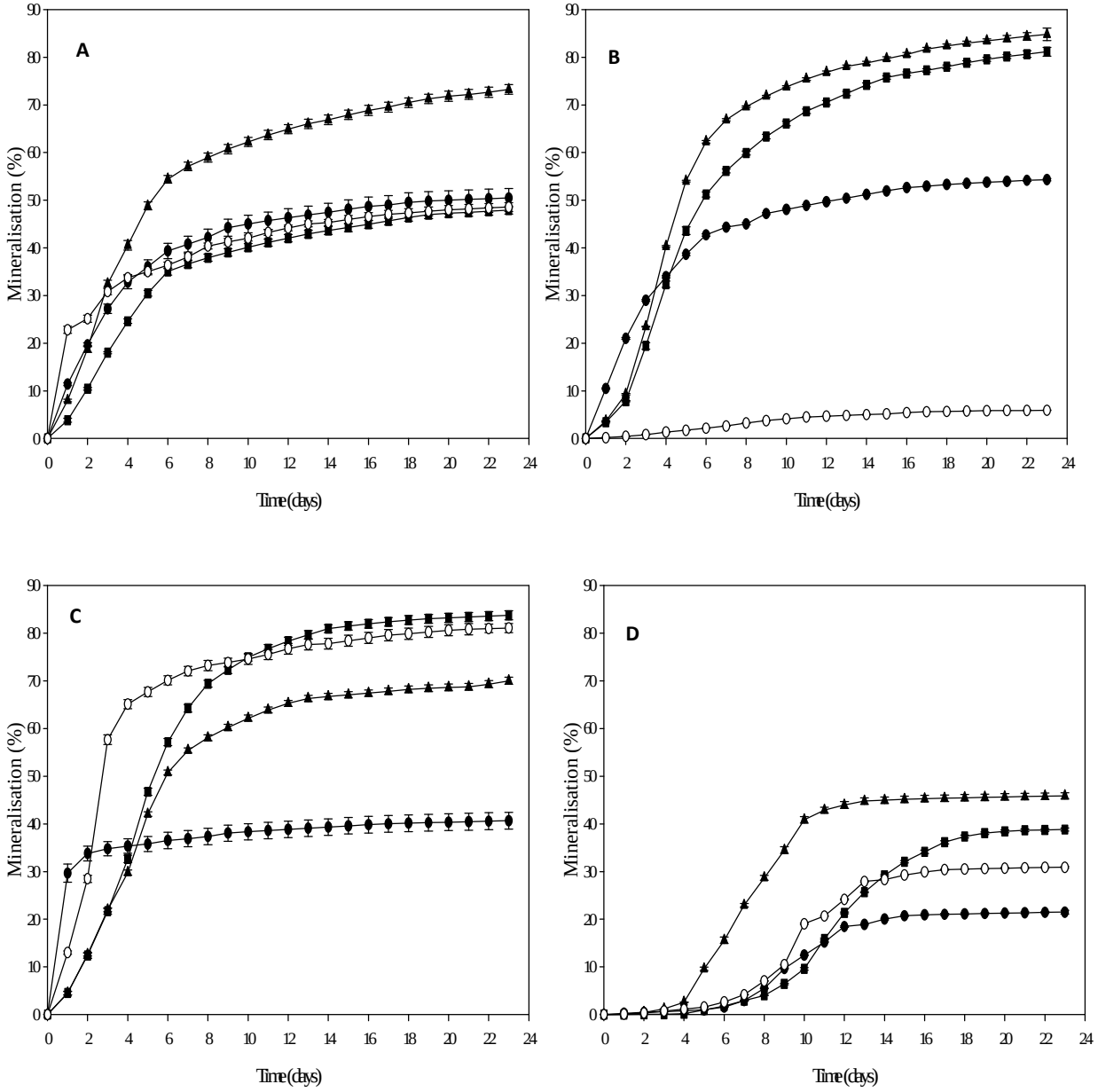


769 Figure 3

770

771

772



773 Table 1: Characteristics and properties of soil A, B and C. Errors are shown as 1 SEM (n=3).

774

Properties	Soil A	Soil B	Soil C
Moisture content (%)	15.60 ± 0.41	22.13 ± 1.56	33.12 ±0.22
Bulk density (kg l^{-1})	0.97	0.82	0.58
pH in water	6.80 ± 0.23	7.47 ± 0.03	6.77 ± 0.09
pH in 0.01 M $CaCl_2$	6.50 ± 0.00	6.60 ± 0.00	6.10 ± 0.06
Organic carbon (%)	6.75 ± 0.20	8.50 ± 2.24	15.39 ± 0.84
Organic matter (LOI %)	11.60 ± 0.34	14.62 ± 3.85	26.47 ± 1.45
DOC (µg ml^{-1})	75.33 ± 12.17	151.67 ± 34.71	133.67 ± 22.45
TOC (µg g^{-1})	167.67 ± 7.62	280.33 ± 67.16	230.33 ± 23.21
Carbon content (%)	8.91 ± 0.48	7.80 ± 0.85	17.50 ± 1.61
Extractable nitrogen content (%)	0	0	0
Extractable phosphorus (%)	0	0	0
Hydrocarbon Fraction (mg/kg)			
Total Aliphatic	15091	19869	7271
EC ≥ 10 – 12	115	915	4379
EC ≥ 12 – 16	11000	14608	2259
EC ≥ 16 – 35	3965	4256	11014
Total Aromatic	5756	9686.1	58
EC ≥ 10 – 12	26	86	1801
EC ≥ 12 – 16	968	1599	3797
EC ≥ 16 – 21	2645	4275	
TPH (mg/kg)	20848	29555	18285
Particle size Analysis:			
Clay (%)	50.61 ± 0.14	43.60 ± 0.23	30.67 ± 1.10
Sand (%)	24.66 ± 0.99	32.12 ± 0.14	49.62 ± 0.24
Silt (%)	24.73 ± 1.07	24.28 ± 0.36	19.67 ± 0.89
Total Heterotrophs (CFU g^{-1})	$5.9E^{05} \pm 7.6E^{04}$	$7.1E^{07} \pm 1.8E^{07}$	$9.8E^{06} \pm 1.3E^{06}$
Total degraders (CFU g^{-1}):			
- Diesel	$1.1E^{03} \pm 5.8E^{03}$	$9.9\ E^{06} \pm 2.6E^{06}$	$3.3E^{05} \pm 1.0E^{05}$
- Hexadecane	$1.0E^{05} \pm 1.0E^{03}$	$1.3E^{04} \pm 4.1E^{04}$	$1.0E^{05} \pm 1.0E^{04}$
- Octacosane	$3.4E^{04} \pm 1.5E^{03}$	$6.7E^{04} \pm 5.8E^{04}$	$1.1E^{05} \pm 4.7E^{04}$
- Phenanthrene	$1.8E^{04} \pm 2.9E^{03}$	$4.8E^{05} \pm 1.3E^{05}$	$8.3E^{04} \pm 2.9E^{04}$
- Pyrene	$1.1E^{04} \pm 3.2E^{03}$	$4.8E^{05} \pm 1.3E^{05}$	$1.3E^{04} \pm 2.9E^{03}$

775

776

777

778 Table 2. Lag phase, rate and overall extent of ^{14}C-hydrocarbons mineralised over 23 d in non-treated

779 and treated soil A. Errors are 1 SEM (n=3).

Soil Treatment	Lag phase (h)	Maximum rate of ^{14}C mineralisation (% h $^{-1}$)	Overall extent of ^{14}C mineralisation (%)
		Hexadecane	
None	179.12 ± 0.60	0.21 ± 0.01	36.94 ± 0.53
Nutrient	24.67 ± 0.46	0.56 ± 0.02	80.77 ± 0.67
Degrader	34. 42 ± 3.73	0.46 ± 0.02	78.73 ± 0.25
Nutrient + degrader	31.03 ± 2.70	0.18 ± 0.01	32.65 ± 0.40
		Octacosane	
None	133.38 ± 7.50	0.54 ± 0.04	54.93 ± 1.30
Nutrient	28.84 ± 0.21	0.46 ± 0.01	79.47 ± 0.94
Degrader	31.01 ± 0.54	0.38 ± 0.01	64.18 ± 0.65
Nutrient + degrader	305.26 ± 3.02	0.05 ± 0.00	8.34 ± 0.08
		Phenanthrene	
None	21.72 ± 0.28	1.11 ± 0.04	43.68 ± 1.80
Nutrient	38.90 ± 1.42	0.47 ± 0.01	45.25 ± 0.95
Degrader	90.49 ± 2.05	0.22 ± 0.00	27.37 ± 0.72
Nutrient + degrader	27.92 ± 0.24	1.02 ± 0.01	82.45 ± 0.80
		Pyrene	
None	294.94 ± 0.32	0.10 ± 0.00	17.81 ± 0.05
Nutrient	195.79 ± 0.86	0.08 ± 0.00	16.76 ± 0.36
Degrader	175.14 ± 2.61	0.11 ± 0.00	21.64 ± 0.16
Nutrient + degrader	400.72 ± 4.47	0.05 ± 0.00	10.59 ± 0.32

780

781

782

783

784

785

786

787

788

789

790

791

792

793

794

795

796 Table 3. Lag phase, rate and overall extent of ^{14}C-hydrocarbons mineralised over 23 d in non-treated

797 and treated soil B. Errors are 1 SEM (n=3).

798

Soil Treatment	Lag phase (h)	Maximum rate of ^{14}C mineralisation (% h $^{-1}$)	Overall extent of ^{14}C mineralisation (%)
Hexadecane			
None	40.07 ±0.15	0.35 ± 0.02	20.00 ± 0.66
Nutrient	72.50 ± 2.25	0.25 ± 0.01	27.65 ± 0.70
Degrader	87.96 ± 2.88	0.27 ± 0.02	29.01 ± 1.05
Nutrient + degrader	52.18 ± 0.78	0.10 ± 0.00	22.82 ± 0.29
Octacosane			
None	47.74 ± 0.73	0.76 ± 0.00	45.84± 0.68
Nutrient	70.48 ± 2.83	0.26 ± 0.02	35.56 ± 0.66
Degrader	93.56 ± 2.58	0.15 ± 0.00	33.65 ± 0.79
Nutrient + degrader	273.65 ± 9.52	0.03 ± 0.00	7.38 ± 0.14
Phenanthrene			
None	9.03 ± 0.68	1.12 ± 0.09	45.23 ± 1.92
Nutrient	21.61 ± 1.23	0.60 ± 0.01	58.78 ± 1.06
Degrader	21.67 ± 1.17	0.35 ± 0.02	43.31 ± 0.27
Nutrient + degrader	7.64 ± 0.09	0.93 ± 0.01	79.92 ± 0.16
Pyrene			
None	162.18 ± 0.65	0.29 ± 0.01	30.80 ± 0.07
Nutrient	104.84 ± 0.65	0.66 ± 0.01	61.32 ± 0.92
Degrader	109.21 ± 0.16	0.52 ± 0.00	56.67 ± 0.06
Nutrient + degrader	161.40 ± 0.58	0.27 ± 0.01	41.10 ± 0.36

799

800

801

802

803

804

805

806

807

808

809

810

811

812

Table 4. Lag phase, rate and overall extent of ^{14}C-hydrocarbons mineralised over 23 d in non-treated and treated soil C. Errors are 1 SEM (n=3).

Soil Treatment	Lag phase (h)	Maximum rate of ^{14}C mineralisation (% h $^{-1}$)	Overall extent of ^{14}C mineralisation (%)
		Hexadecane	
None	9.96 ± 0.28	1.00 ± 0.03	50.48 ± 2.00
Nutrient	28.18 ± 1.42	0.32 ± 0.00	48.60 ± 0.82
Degrader	15.08 ±0.45	0.57 ± 0.01	73.26 ±1.01
Nutrient + degrader	5.27 ± 0.17	0.85 ±0.03	48.55 ± 0.93
		Octacosane	
None	11.04 ± 0.07	0.91 ± 0.01	54.32 ± 0.25
Nutrient	32.67 ± 0.90	0.54 ± 0.00	81.17 ± 0.93
Degrader	29.71 ± 0.79	0.70 ± 0.01	84.82 ± 1.31
Nutrient + degrader	338.13 ± 8.79	0.03 ± 0.00	5.91 ± 0.07
		Phenanthrene	
None	4.23 + 0.15	2.37 ±0.08	40.67 ± 1.77
Nutrient	25.43 ± 1.3	0.59 ± 0.01	83.76 ±1.19
Degrader	25.44 ±1.18	0.51 ± 0.01	70.02 ±0.75
Nutrient + degrader	9.24 ± 0.25	1.22 ± 0.02	81.08 ± 0.94
		Pyrene	
None	170.92 ±0.39	0.19 ± 0.00	21.48 ± 0.41
Nutrient	195.66 ± 0.22	0.26 ± 0.02	38.81 ± .31
Degrader	104.31 ± 0.36	0.31 ± 0.02	45.89 ± 0.68
Nutrient + degrader	175.01 ± 0.19	0.36 ± 0.00	30.90 ± 0.19