International Biodeterioration & Biodegradation, Volume 160, May 2021, Article number 105227
DOI:10.1016/j.ibiod.2021.105227

TEMPORAL CHANGES IN THE EXTRACTABILITY, BIOACCESSIBILITY AND BIODEGRADATION OF TARGET HYDROCARBONS IN SOILS FROM FORMER REFINERY FACILITIES

Marcie G. Towell [1], Gabriela M. Vázquez-Cuevas [1,2], Jessica Bellarby [3], Graeme I. Paton [3], Frédéric Coulon [4], Simon J. T Pollard [4]and Kirk T. Semple [1]*

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

[2]Área Académica de Biología, Instituto de Ciencias Básicas e Ingeniería / Parque Científico y Tecnológico, Universidad Autónoma del Estado de Hidalgo, 42010, Hidalgo, Mexico.

[3]School of Biological Sciences, University of Aberdeen, Aberdeen AB24 3UU, UK

[4]School of Water, Energy and Environment, Cranfield University, Cranfield MK430AL, UK

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

Abstract

This study investigated the extractability, bioaccessibility and biodegradation of ^{14}C-phenanthrene and ^{14}C-octacosane in two soils from former oil refinery facilities over 341 days. The impact of biostimulation and bioaugmentation treatments was also evaluated. At 0, 31, 62, 124 and 341 days, the loss and extractability (using dichloromethane, methanol:water and hydroxypropyl-β-cyclodextrin (HPCD)) of the ^{14}C-hydrocarbons were measured . Further at each time point, the mineralisation of the ^{14}C-hydrocarbons was measured respirometrically under the different conditions. In general, extractions with methanol: water and HPCD were similar for both hydrocarbons in the different treatments; however, these values were less that those measured with DCM. Overall, significantly higher ($p \leq 0.05$) amounts of ^{14}C-phenanthrene were lost, readily extracted and mineralised in the soils, with treatments having little impact upon the degradation of this hydrocarbon over 341 days. Conversely, bioaugmentation significantly increased the loss of ^{14}C-octacosane residues from soils and sustained degradation after 31 days. Surprisingly, HPCD and methanol: water both under-predicted the extent to which the contaminants were degraded at each time point. Determining the likelihood of effective biodegradation by the stimulation of indigenous microorganisms or through bioaugmentation needs to be assessed by both chemical and biological measurements of bioaccessibility, rather than just by that which is totally extractable from soil. However, soils which have high loadings of organic matter and/or organic contaminants may prevent accurate assessment of contaminant bioaccessibility, as measured by HPCD.

Keywords: Phenanthrene, octacosane, bioaccessibility, mineralisation, bioaugmentation, biostimulation

1. Introduction

The ubiquitous presence of weathered petroleum hydrocarbons in soil, comprising both long-chained aliphatic hydrocarbons and medium to heavy molecular weight polycyclic aromatic hydrocarbons (MMW and HMW PAHs), remains a major concern due to their environmental persistence, toxic characteristics and putative impact (Babu et al., 2019). Accordingly, there is considerable interest surrounding the fate and behaviour of petroleum hydrocarbons in soil, with particular attention directed towards their remediation (Samanta et al., 2002; Tripathi et al., 2015). Numerous studies have shown that microbial degradation is a key removal process of petroleum-derived hydrocarbons from soil, with hydrocarbon loss via physico-chemical processes limited by their recalcitrant molecular structure, high hydrophobicity and ability to partition strongly within the soil matrix (Macleod and Semple, 2002; Cipullo et al., 2018).

Predominantly, the degradation potential of petroleum hydrocarbon contaminated soils will be dependent upon hydrocarbon bioaccessibility and the catabolic ability of the microbial community (Riding et al., 2013; Varjani and Upasani, 2017). Specific important parameters impacting biodegradation include hydrocarbon physical-chemical properties; soil pH, moisture, soil organic matter content, nutrient availability and the size, composition and degrading microbial activity (Leahy and Colwell, 1990; Varjani, 2017). Furthermore, as petroleum hydrocarbons weather and undergo interactions with soil components, their bioaccessibility will decrease due to the process of ‘aging’ and the formation of bound residues (Duan et al., 2015a). Specifically, sequestration via sorption and diffusion into/onto soil constituents is a major mechanism controlling the ageing process, and is suggested to be strongly influenced by contaminant physico-chemical characteristics including aqueous solubility, hydrophobicity, octanol:water partition coefficient (K_{ow}) and molecular structure (Rhodes et al., 2010b; Duan et al., 2015a; Yu et al., 2016).

^{14}C-Labelled hydrocarbons may be utilised as a mean to trace their fate and behaviour in complex soil environments under reproducible and controlled conditions (Ortega-Calvo et al., 2013). Furthermore, harsh and exhaustive extraction techniques using solvents, such as dichloromethane (DCM), can be employed to quantify total ^{14}C-hydrocarbon concentrations in soil (Riding et al., 2013). However, DCM extraction measurements have been shown to often overestimate contaminant bioaccessibility in soil, and as such may poorly reflect hydrocarbon biodegradation and bioremediation potential, as well as associated risk (Alexander, 2000; Riding et al., 2013; Adedigba et al., 2018). Conversely, it has been suggested that measurements of the HPCD and methanol:water extracted fractions, as well as the measurement of the mineralisation of ^{14}C-hydrocarbons may provide a more realistic indication of hydrocarbon bioaccessibility and degradability in soils (Patterson et al., 2004; Dew et al., 2005; Adedigba et al., 2018).

Although widely documented, there are conflicting reports about the use of biostimulation (nutrient addition) and bioaugmentation (addition of a microbial degrader) as a mean to stimulate catabolic activity, enhance degradation and facilitate hydrocarbon removal from contaminated soil (Towell et al., 2011b; Chen et al., 2015; Jiang et al., 2016). Furthermore, the bioremediation process is often limited by the presence of complex weathered hydrocarbon mixtures, which may influence soil-contaminant–microbe interactions, reflected on the rate and extent at which individual hydrocarbons and further contamination episodes are degraded.

Phenanthrene and octacosane were selected as representatives of PAHs and long-chained aliphatic hydrocarbons, respectively, as they are common petroleum constituents and are found in oil-contaminated soils. The aims of this study were to (a) investigate temporal changes in the loss, chemical extractability and degradation of ^{14}C-phenanthrene and ^{14}C-octacosane in two genuinely contaminated soils from former refinery facilities, and (b) assess

the impact biostimulation and bioaugmentation treatments have on the fate, behaviour and bioaccessibility of these hydrocarbons, over 341 d. The study also highlighted the limitations of HPCD as a chemical measure of contaminant bioaccessibility in soil with high level of organic matter and hydrocarbon contamination. Furthermore, the impact that biostimulation and bioaugmentation towards genuinely contaminated soil from a former refinery facility will provide realistic information regarding the application of these well-known approaches when dealing with soil affected by complex mixtures of hydrocarbons under natural conditions. To the authors knowledge, there is a paucity of information on the behaviour, biodegradation and bioaccessibility of phenanthrene and octacosane in genuinely contaminated soils over an extended time frame.

2. Materials and methods

2.1 Soil and contaminant characteristics

Two soils genuinely contaminated with weathered hydrocarbons were collected (top soil; 5-20 cm) from former oil refinery facilities. The soils were labelled as A and B (to maintain owner anonymity), and identified as a clay and sandy loam, respectively. Soil A had previously undergone biopiling treatment, whereas soil B was untreated. The soils were air-dried (24 h) and passed through a 2 mm sieve to remove stones, plant material and facilitate mixing. Prior to spiking, the soils physico-chemical properties were analysed (n = 3) following the methods described by Towell *et al.* (2011a) and are presented in Table 1. In short, soil moisture and loss on ignition (LOI) organic matter contents were determined through oven drying at 105°C and combustion in a furnace at 450°C, respectively, for 24 h. Soil pH was measured in a 1:5 soil (dry wt) to liquid suspension with 0.01 M calcium chloride ($CaCl_2$) solution and then separately using deionised water. Soil organic carbon

(OC) content was determined via elemental analysis on a Carlo Erba CHNS- OEA 1108 CN-Elemental Analyser. Acetic acid-extractable phosphorus was measured by shaking soil (0.5 ± 0.1 g, dry wt) with 2.5% acetic acid (40 ml), followed by filtration through Whatman 44 filter paper. For extractable ammonium and nitrogen, soil was extracted with 1M KCl. Resultant solutions were then analysed on a flow injection analyser (FIAstar). Total heterotrophic and degrading microbial numbers were enumerated through measurement of colony forming units (CFUs g^{-1} soil, dry wt) on plate count agar and minimal agar supplemented with 0.1% diesel, octacosane or phenanthrene. In brief, soil (1.0 ± 0.1 g, dry wt) was extracted with quarter strength Ringer's solution (10 ml) and, 1 ml of the extract serially diluted with Ringer's solution (0.9 ml). The resultant solutions (0.1 ml) were then spread evenly over agar plates and incubated in the dark at 25 ± 1°C. Colony forming unit counts were performed at 4, 7, 10 and 15 d following agar inoculation.

Quantification of total petroleum hydrocarbon (TPH) concentrations in each soil was performed using sequential ultrasonic solvent extraction (Risdon et al., 2008). TPH aliphatic and aromatic fractions were assessed through gas chromatography-mass spectrometry (GC-MS) 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 1 µl was applied. The oven temperature was ramped from 60°C to 220°C (at 20°C min^{-1}), then to 310°C (at 6°C min^{-1}) and held at this temperature (for 15 min). For quantitative analysis of target alkanes and PAHs the mass spectrometer was operated using the full scan mode (range *m/z* 50-500). Each hydrocarbon 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} (alkanes) and from 1 to 5 µg ml^{-1} (PAHs). Internal standards for the

alkanes were nonadecane-d_{40} and Triacontane-d_{62} and Naphthalene d_8, Phenanthracene-d_{10}, Chrysene-d_{12} and Perylene d_{12}. Controls, a 500 µg ml^{-1} diesel standard and mineral oil, were analysed every 10 samples. Duplicate reagent control (containing no soil) and reference material were also systematically used. The reference material was an uncontaminated soil of known characteristics, spiked with a diesel and mineral oil standard at a concentration equivalent to 16,000 mg kg^{-1}.

2.2 Soil spiking and microcosm preparation

Soils A and B (3.6 kg dry wt) were rehydrated with deionised water to 60% water holding capacity (WHC). One aliquot of each soil (1.8 kg dry wt) was then spiked with $^{12/14}$C-octacosane or $^{12/14}$C-phenanthrene to deliver a ^{12}C-hydrocarbon concentration of 50 mg kg^{-1} and ^{14}C-activity of 83 kBq kg^{-1} soil (dry wt). Octacosane and phenanthrene standards were prepared using acetone as a carrier (20 ml) and spiked into soil following the spoon and bowl method proposed by Doick *et al.* (2003). Following spiking, soil aliquots were divided into three portions (600 g dry wt) and adjusted to give the following: (i) $^{12/14}$C-hydrocarbon spiked only, which served as controls, (ii) biostimulated treatment (nutrient amended) and (iii) bioaugmented treatment (amended with a microbial inoculum).

Nutrient amendment comprised nitrogen and potassium added to soil aliquots to give a C:N:P soil ratio of 100:10:1; where the carbon content of soils A and B was calculated (measured TPH + 50 mg kg^{-1} ^{12}C-hydrocarbon added) to be 32 mg and 20.2 mg g^{-1} (dry wt) soil, respectively. Nitrogen was added using a 5 M ammonium nitrate solution (13.6 ml and 8.6 ml per 600 g dry wt soil A and B, respectively). Potassium was added via a 1 M potassium phosphate buffer (pH 7, 7.6 ml and 4.8 ml per 600 g dry wt soil A and B), prepared using anhydrous potassium orthophosphate and anhydrous dipotassium orthophosphate. For the

bioaugmentation treatment, a commercial mixed hydrocarbon degrader inoculum (Remedios Limited, UK; inoculum composition unknown) able to utilise octacosane and phenanthrene as a carbon growth source was cultured in autoclaved Bushnell-Haas medium (3.27 g l^{-1}) on an orbital shaker (100 rpm) at 20 ± 1°C. Bushnell-Haas medium was prepared with deionised water, supplemented with 1% ethanol and 1000 mg l^{-1} salicylic acid. After 2 d incubation, the inoculum was added to soil (6 ml per 600 g soil dry wt) to give 10^6 cells g^{-1} dry wt soil. Cells were enumerated through measurement of CFUs on plate count agar, as described previously.

Following amendment, the moisture content of all soil treatments (600 g dry wt) was adjusted to 80% WHC using deionised water. Soil treatments were then weighed into amber glass jars (200 g dry wt soil, n = 3) and 'aged' in the dark at 21±1°C for the duration of the study. An additional set of microcosms (n = 3) consisting of rehydrated soil (200 g dry wt) in amber jars were also prepared as analytical blanks. After 0, 31, 62, 124 and 341 d of incubation, a moisture check was performed on microcosms and soil treatments were sampled and processed as described in the following sections.

2.3 Determination of total ^{14}C-associated activity in soils

The soils were not sterilised in order to avoid changes within its biological and physicochemical properties such as SOM (Wang et al., 2014) and therefore the partitioning of the contaminants (Ortega-Calvo et al., 2015). Quantification of the total ^{14}C-associated activity was evaluated at every time point, thereby allowing the measurement of differences in the loss of the ^{14}C-contaminants in the control and treatment incubations. At each aging period, total ^{14}C-octacosane or ^{14}C-phenanthrene associated activity remaining in soil treatments was determined via sample oxidation. Samples were combusted (3 min) on a Packard 307 Sample Oxidiser (1.0 g ± 0.1 g wet wt soil + 200 µl combustaid; n = 3).

Permafluor-E (10 ml) was utilised as a scintillation cocktail and Carbosorb-E (10 ml) to trap released $^{14}CO_2$. Prior to sample combustion, trapping efficiency of the sample oxidiser was determined to be >95%. Following storage in the dark for 12 h (to reduce the effects of chemi-luminescence), ^{14}C-activity was quantified by liquid scintillation counting (LSC, Canberra Packard Tri-Carb 2250CA, UK), using blank and quench correction techniques.

2.4 Quantification of extractable ^{14}C-octacosane and ^{14}C-phenanthrene associated activity from soils

Extractable fractions of ^{14}C-hydrocarbons were evaluated through three different approaches, where each one can be used as a predictor of the behaviour of the hydrocarbon based on its bioaccessibility (Riding et al., 2013). DCM was used as an exhaustive technique to quantify total extractability/non-extractable hydrocarbon fractions, whilst methanol:water and HPCD represented less aggressive extractions to determine readily extractable and bioaccessible fractions of the ^{14}C-hydrocarbons (Semple et al., 2013; Vázquez-Cuevas and Semple, 2016). At each time point, the extractability of ^{14}C-octacosane or ^{14}C-phenanthrene associated activity in soil treatments was determined by dichloromethane (DCM), methanol:water (1:1 v/v) and hydroxypropyl-β-cyclodextrin (HPCD) shake extractions (n = 3). To measure DCM extractability, soil treatments (1.5 g ± 0.1 g wet wt) were grounded with sodium sulphate (8 ± 0.1 g) to dry the soil and facilitate DCM-soil interactions. Samples were then transferred into 50 ml Teflon centrifuge tubes and 25 ml DCM added to each tube. Methanol:water extraction was performed following the method of Macleod & Semple (2003), where soil treatments (8 g ± 0.1 g dry wt) were weighed into Teflon centrifuge tubes (50 ml) and 24 ml methnol:water (1:1 v/v) added to each tube. For HPCD extraction, soil treatments (1.25 g ± 0.1 g wet wt) were mixed with 25 ml 50 mM HPCD solution in 50 ml Teflon centrifuge tubes as per

Vázquez-Cuevas & Semple (2016). Tubes were then sealed and all the suspensions shaken for 22 h at 100 rpm on an orbital shaker. Following shaking, soil and supernatants were separated by centrifugation at 3000 x *g* for 1 h. Supernatants for DCM (5 ml), methanol:water (3 ml) and HPCD (6 ml) extractions were sampled, added to Goldstar liquid scintillation cocktail (15 ml) and then counted by LSC as described previously. A mass balance of ≥ 98% was calculated on extraction completion through combustion of extracted soil pellets (1 ± 0.1 g) using sample oxidation following the previously described methodology.

2.5 Mineralisation of ^{14}C-octacosane and ^{14}C-phenanthrene in soils

Mineralisation assays based on the soil slurry respirometric approach (Reid et al., 2001; Semple et at., 2006; Vázquez-Cuevas and Semple, 2016) were selected to assess the impact of the treatments towards microbial catabolic response. At each aging period, the mineralisation of remaining ^{14}C-octacosane or ^{14}C-phenanthrene in soil treatments was assessed using respirometric assays as described by Stroud *et al.* (2007b). Respirometric assays (n = 3) were performed in modified 250 ml Schott bottles prepared with 10 g ± 0.2 g soil (wet wt) and 30 ml sterile minimal basal salts medium (MBS). Glass vials (7 ml) containing 1 M NaOH (1 ml) were suspended from the respirometer Teflon-lined lids to trap released $^{14}CO_2$ formed during ^{14}C-hydrocarbon mineralisation. Respirometers containing non-spiked rehydrated soil were also prepared as analytical blanks. The respirometers were then shaken onto an orbital shaker at 100 rpm and 21 ± 1°C, in the dark. Every 24 h over a period of 20 d respirometer $^{14}CO_2$ traps were replaced and Ultima Gold scintillation cocktail (5 ml) added to the sampled vial. Samples were quantified by LSC as previously described.

2.6 Statistical analysis

Following blank correction, statistical analysis of the results was performed using SigmaStat for Windows (Version 2.03 SPSS). Significant effects of treatments (biostimulation and bioaugmentation), hydrocarbon type (phenanthrene and octacosane), and aging time (0, 31, 62, 124 and 341 days) on the loss, chemical extractability and mineralisation parameters (lag time, maximum rates and overall extents) of the two ^{14}C-hydrocarbons were evaluated using ANOVAs (Tukey Test) and/or Student t-tests at a 95% confidence level ($p \leq 0.05$). Graphs were presented using SigmaPlot 2000 for Windows (Version 10.0, SPSS Inc.).

3. Results

3.1 Loss of ^{14}C-phenanthrene and ^{14}C-octacosane residues from soil

Loss of ^{14}C-activity from the soils was assessed at each time point for both of the hydrocarbons under control and treatment regimens (Tables 2 and 3; SM-1). The greatest losses of ^{14}C-phenanthrene was observed in the control and biostimulated treatments for soil A and B, respectively. Total ^{14}C-phenanthene associated activity remaining in control soils decreased by 79 % (soil A) and 55% (soil B) between 0 and 341 d. The initial rapid rate of loss of ^{14}C-residues was 1.03 % d^{-1} within the first 62 days, followed by a slower rate of loss of 0.01 % d^{-1} (62 – 341 d). Although losses of ^{14}C-activity were observed to be significantly higher in the control soil A than B, no significant differences ($p \geq 0.05$) in the rate and loss of ^{14}C-phenanthrene residues were apparent (Table 2). Furthermore, bioaugmentation had a negative impact upon the loss of ^{14}C-phenanthrene residues from both soils, with total ^{14}C-phenanthrene activity remaining predominantly, but not exclusively, significantly higher ($p \leq 0.05$) in bioaugmented than control soils.

In the case of ^{14}C-octacosane, after 341 d incubation associated residues from control soils reduced to 37 and 38 % in soils A and B, respectively (Table 3). Overall, trends showed a rate of ^{14}C loss of 0.57 % d^{-1} within the first 62 d, followed by a slower rate of loss of 0.1 % d^{-1} from day 62 until the end of the experiment. Furthermore, there was no significant difference in the loss kinetics of ^{14}C-octacosane residues between controls soils A and B; total ^{14}C-octacosane associated activity remaining was predominantly, but not exclusively, statistically similar ($p \geq 0.05$) at the different aging times (Tables 2 and 3). Contrary to the observed for phenanthrene, bioaugmentation significantly increased ($p \leq 0.05$) the loss of ^{14}C-octacosane residues from soils, with differences in the rate and extent of ^{14}C-octacosane loss (Table 3). Amounts of ^{14}C-octacosane associated activity remaining in bioaugmented soils were significantly lower ($p \leq 0.05$) at 31, 62, 124 and 341 d than the controls. The impact of bioaugmentation treatment was observed to be more pronounced in soil A, with ^{14}C-octacosane activity reducing to 4 % (33% lower than the control), as opposed to 24% (14% lower than the control) in soil B, at 341 d. Furthermore, total ^{14}C-octacosane activity remaining in the soil was significantly lower ($p \leq 0.05$) in bioaugmented soil A than B, at all aging times (Table 3).

Significant losses ($p \leq 0.05$) of ^{14}C-phenanthrene and ^{14}C-octacosane associated residues were observed for both control soils following a biphasic behaviour characterised by an initial short period of rapid loss. Although soil was observed to have an important initial catabolic activity, bioaugmentation and nutrient treatments had a varying impact upon the loss of ^{14}C-phenanthrene and ^{14}C-octacosane residues from soils (Tables 2 and 3; SM-1).

3.2 Extractability of ^{14}C-phenanthrene and ^{14}C-octacosane associated activity from soils

In this study, DCM, methanol:water and HPCD shake extractions were utilised to determine total extractable and readily extractable hydrocarbon fractions, respectively (Table 2 and 3). Over 341 d, the amounts of ^{14}C-phenanthrene extracted by DCM showed that nutrient and bioaugmentation treatments had little impact ($p > 0.05$) on their extractability (Table 2). Overall, significantly higher ($p \leq 0.05$) extents of ^{14}C-phenanthrene associated activity were consistently extracted from soils by DCM (exhaustive extraction), as opposed to methanol:water and HPCD extracted (Table 2). During the course of the experiment (341d), extracted amounts of ^{14}C-phenanthrene decreased by 14 and 20% (controls), 11 and 22 % (nutrient treated) and 13 and 23% (bioaugmented) for soils A and B, respectively (Table 2). Moreover, after 124 and 341 d incubation, significantly less ($p \leq 0.05$) ^{14}C-phenanthrene was consistently extracted from soil B treatments and controls, in respect to soil A (Table 2). When looking at the readily extractable fractions of ^{14}C-phenanthene (Table 2), results showed that between 0 and 341 d, extraction by methanol:water and HPCD significantly decreased ($p \leq 0.05$) by ≥15 and 19% for soil A and ≥13 and 31% for soil B control and treatments, respectively. Between soils, significantly less ($p \leq 0.05$) ^{14}C-phenanthrene associated activity was consistently extracted (using methanol:water and HPCD) from soil B, suggesting phenanthrene bioaccessibility may be lower in this soil (Table 2). It is important to note that both the soils used in this study contained different OM/OC concentrations, with soil B containing 11.08 and 6.89% more OM/OC (Table 1).

For the biostimulation and bioaugmentation treatments, overall, no significant trends ($p \geq 0.05$) in ^{14}C-phenanthrene extraction (by methanol:water and HPCD) were determined between control and treated soil B, and control and nutrient treated soil A (Table 2). However, for bioaugmented soil A, ^{14}C-phenanthrene extraction by methanol:water was significantly lower ($p \leq 0.05$) and HPCD-extraction higher than the control at 62, 124 and

341 d (Table 2). Due to the conflicting nature of these extraction results (between extraction types, and with the mineralisation findings of this study), the cause for these observations remains unclear.

As in the case of ^{14}C-octacosane, total extractable fraction (DCM extracted) decreased by 5 and 30 % (controls), 7 and 16% (nutrient treated) and 24 and 21% (bioaugmented) for soils A and B, respectively (Table 3). Similar to the observed for ^{14}C-phenanthrene, about 40% less ^{14}C-octacosane was extracted from soil B, in respect to soil A, at these aging times at longer incubation times (124 – 341 d). Nutrient and bioaugmentation treatments had a varying impact on the DCM-extractability and aging of ^{14}C-octacosane in soils (Table 3). In soil B, nutrients increased the DCM-extractability of ^{14}C-octacosane (≥ 9% higher than the controls) at all ageing times (> 0 days) and no significant impact on its DCM-extractability ($p \geq 0.05$) (Table 3). In soil A, bioaugmentation significantly decreased ($p \leq 0.05$) ^{14}C-octacosane extraction (compared to controls), at all ageing time points (> 0 days), whilst biostimulation had no effect on DCM-extractability (Table 3). Furthermore, bioaugmentation appeared to increase the magnitude of the aging of ^{14}C-octacosane in this soil. Between 0 and 341 d ageing, DCM-extractability decreased by 23.89% for bioaugmented soil, as opposed to 8.14% for the control.

On the other hand, methanol:water and HPCD-extractability of ^{14}C-octacosane were significantly lower ($p \leq 0.05$) than ^{14}C-phenanthrene for both soils (Table 3). For example, at 31 d, ^{14}C-octacosane extraction by methanol:water ranged from 1-8% for soils (control and treated), as opposed to 39-59% for ^{14}C-phenanthrene. Overall, biostimulation had no significant impact ($p \geq 0.05$) on the HPCD-extractability of ^{14}C-octacosane in soils (Table 3). Conversely, bioaugmentation generally increased HPCD-extractability, in respect to controls, and with aging time. As bioaugmentation also increased the mineralisation of ^{14}C-octacosane in soils after 31 d, these findings may be attributed to the catabolic activity of specialised

hydrocarbon degraders influencing the desorption kinetics of ^{14}C-octacosane from the sorbed to the aqueous phase.

Overall, results showed that with increasing soil incubation time, the DCM-extractability of ^{14}C-phenanthrene and ^{14}C-octacosane significantly decreased ($p \leq 0.05$), indicating that aging was taking place (SM-1 and SM-2). In addition, and as expected, significantly lower ($p \leq 0.05$) amounts of ^{14}C-phenanthrene and ^{14}C-octacosane were extracted from soils by methanol:water and HPCD (Table 2 and 3).

3.3 ^{14}C-Phenanthrene and ^{14}C-octacosane biodegradation in soils

Mineralisation of ^{14}C-hydrocarbons from soil was assessed at each time point as an indication of the potential of the treatments promoting the biodegradation of each hydrocarbon. In this study, differences in the mineralisation of ^{14}C-hydrocarbons were apparent between aromatic and aliphatic hydrocarbons, and soil types, with biostimulation and bioaugmentation having a varying impact upon hydrocarbon degradation (Tables 4 and 5; SM-2 and SM-3). The indigenous microflora of both soils demonstrated high catabolic potential to degrade ^{14}C-phenanthrene with ≥40% mineralised over the aging period in the control soils. This is an expected response given the exposure history of the indigenous microflora to high aromatic hydrocarbon concentrations (Table 1).

When analysing the behaviour of both soils across time, at 0 d significantly less ($p \leq 0.05$) ^{14}C-phenanthrene was mineralised in soil B, compared to soil A (Table 4; SM-2). At this stage (0 d ageing), lag times were also significantly longer ($p \leq 0.05$) and maximum rates of ^{14}C-phenanthrene mineralisation lower for soil B. For instance, while soil A had a lag phase of 28.76 h in soil B took 42.48 h to mineralise >5% of the ^{14}C-activity in the control soil. This trend was not observed over the whole aging period, with maximum rates and extents of

^{14}C-phenanthrene mineralisation between control soils often being statistically similar ($p \geq 0.05$) at subsequent ageing times (Table 4; SM-2). In this study, soil A contained considerably higher TPH (1.6 times more) and phenanthrene concentrations (798 mg kg^{-1}), and had a larger phenanthrene degrading population than soil B (Table 1). These observations are also supported by^{14}C-phenanthrene lag times remaining significantly longer ($p \leq 0.05$) for soil B (than soil A).

For the aliphatic hydrocarbon, comparisons between ^{14}C-mineralisation parameters (lag times, maximum rates and extents of mineralisation) indicated that ^{14}C-octacosane was less readily degraded by the microflora of both soils (Table 5; SM-3). In general, maximum rates and extents of ^{14}C-octacosane mineralisation were significantly lower ($p \leq 0.05$) compared to ^{14}C-phenanthrene, even though the soil microflora had been previously exposed to high aliphatic hydrocarbon concentrations (≥7271 mg kg^{-1}) (Table 5; SM-3). Similar findings were observed for biostimulated and bioaugmented soils (Table 5; SM-3). Lag times for comparable soil treatments were also often significantly higher ($p \leq 0.05$) for ^{14}C-octacosane, compared to ^{14}C-phenanthrene, further suggesting reduced catabolic behaviour toward this hydrocarbon.

3.3.1 Impact of biostimulation and bioaugmentation

In this study, biostimulation and bioaugmentation treatments generally had no impact upon the degradation of ^{14}C-phenanthrene in soils over 341 d (Table 4; SM-2). ^{14}C-Phenanthrene mineralisation parameters were predominately (but not exclusively) statistically similar ($p \geq 0.05$) between control, nutrient treated and bioaugmented soils, at aging times (Table 4). The exception to this was nutrient treated soil B without ageing (0 d), where lag times were higher and extents/maximum rates of ^{14}C-phenanthrene mineralisation significantly lower ($p \leq 0.05$),

compared to the control (Table 4; SM-2). At 341 d, similar high extents and maximum rates of ^{14}C-phenanthrene mineralisation, and short lag times were determined between control and bioaugmented soils, demonstrating that effective and sustained degradation was achieved by the native microflora of both soils alone (Table 4; SM-2).

Conversely, biostimulation and bioaugmentation significantly increased ($p \leq 0.05$) ^{14}C-octacosane mineralisation in soil A, but generally only after 31 d ageing (Table 5; SM-3). High ^{14}C-octacosane associated catabolic activity was further reflected by lag times being about 8 h shorter for treated soils, than the control, after 31 d ageing. Between treatments, ^{14}C-octacosane mineralisation was often (but not exclusively) significantly higher ($p \leq 0.05$) for bioaugmented soil A, as opposed to nutrient treated (Table 5; SM-3). These findings also correspond with the significantly lower ($p \leq 0.05$) amounts of ^{14}C-octacosane associated activity remaining in bioaugmented soil A (compared to control and nutrient treated), after 341 d. It is suggested that although comparably rapid and extensive mineralisation ($\geq$ 60%) and ^{14}C-octacosane residue loss was attained by the indigenous microflora of soil A, between 0-31 d, the presence of specialised degraders was required to sustain degradation with increasing aging time, and facilitate octacosane removal from this soil.

Similarly, in soil B bioaugmentation significantly increased ($p \leq 0.05$) the rates and extents of ^{14}C-octacosane mineralisation and reduced lag times in respect to controls, at 62, 124 and 341 d (SM-3). Bioaugmentation had considerably less impact on ^{14}C-octacosane degradation in soil B, where extents of ^{14}C-octacosane mineralised were significantly lower ($p \leq 0.05$) for bioaugmented soil B, than A, at aging times (Table 5; SM-3). As per previously discussed these findings may be attributed to the putative impact of OM/OC on the bioaccessibility of ^{14}C-octacosane for degradation purposes in this soil.

3.4 Comparisons between methanol:water and HPCD-extractability and mineralisation of ^{14}C-phenanthrene and ^{14}C-octacosane

In this study the mineralisation and extraction (using methanol:water and HPCD) of ^{14}C-phenanthrene and ^{14}C-octacosane in control and treated A and B soils (genuinely contaminated with high levels of weathered hydrocarbons) was measured over 341 d, and the ratio between total amounts extracted and mineralised compared (Tables 6 and 7).

In general, both methanol:water and HPCD extraction underestimated the mineralisation of ^{14}C-phenanthrene in soils over 341 d. At aging times, total amounts of ^{14}C-phenanthrene mineralised were often significantly higher ($p \leq 0.05$) than amounts extracted (by both methanol:water and HPCD) for treated and control soils. This was further reflected by methanol:water and HPCD extraction:mineralisation ratios for treated and control A and B soils being predominantly higher than 0.80, over the aging period (Table 6). Although, for nutrient treated soil B, both extraction methods were determined to significantly overestimate ($p \leq 0.05$) ^{14}C-phenanthrene mineralisation at 62, 124 and 341 d. The exception to these findings was HPCD extraction for bioaugmented soil A, which was determined to strongly correlate with the ^{14}C-phenanthrene mineralisation values in this soil at 31, 62 and 124 d. Furthermore, over 341 d the average HPCD extraction:mineralisation ratio for bioaugmented soil A was 0.99, as opposed to 0.62 and 0.66 for control and nutrient treated soil A, respectively; and 0.51, 1.55 and 0.41 for control, nutrient treated and bioaugmented soil B.

Similarly, for ^{14}C-octacosane there was much variability in both methanol:water and HPCD extraction:mineralisation ratios for control and treated soils over the aging period, with poor correlation to mineralisation generally observed (Table 7). At 0, 31 and 62 d, both extraction techniques grossly underestimated the degradation of ^{14}C-octacosane with significantly

higher ($p \leq 0.05$) extents mineralised in soils (controls and treated) than extracted at these aging times. Furthermore, between 0-62 d, the average HPCD extraction:mineralisation ratios were 0.29, 020 and 0.32 for control, nutrient treated and bioaugmented soil A, respectively, and 0.46, 0.34 and 0.62 for soil B counterparts. Interestingly, at 0 and 31 d, both methanol:water and HPCD extraction:mineralisation ratios for ^{14}C-octacosane were consistently less close to 1 than those determined for ^{14}C-phenanthrene, indicating lower extraction efficiency for this hydrocarbon.

4. Discussion

4.1 Loss of ^{14}C-phenanthrene and ^{14}C-octacosane residues from soil

Loss of both ^{14}C-phenanthrene and ^{14}C-octacosane can be attributed to an initial large and catabolically active indigenous hydrocarbon degrading population present in each of the soils. Several studies have reported significant loss of ^{14}C-hydrocarbons in soils previously contaminated with petroleum hydrocarbons, as opposed to pristine soils, due to prior exposure and adaptation of native soil microflora to use the hydrocarbons as their source of carbon (Okere *et al.*, 2017; Vázquez-Cuevas *et al.*, 2018). In addition, it is proposed that ^{14}C-hydrocabon loss via volatilisation and leaching was minimal due to their low vapour pressure and water solubility, and high hydrophobicity and octanol:water partitioning coefficients (Duan *et al.*, 2015b).

Results also showed that the loss of ^{14}C-phenanthrene and ^{14}C-octacosane residues followed a biphasic curve for both soils with a short period of rapid loss. These findings are also comparable with several studies which have demonstrated that hydrocarbon biphasic loss phases are resultant from rapid degradation controlled by the microbial activity, followed by slow degradation limited by the mass transfer of hydrocarbons to the aqueous phase

(Swindell and Reid, 2006; Rhodes et al., 2010a; Masy et al., 2016). Other causes of this behaviour can be related to a shift within the microbial population (Zhen et al., 2021), which has been previously reported to result on the modification of the biodegradation patterns. More importantly, this microbial succession process could also indicate that with longer treatment protocols more capable degrading consortiums could modify the fate of hydrocarbons in the soil.

As for the specific case of bioaugmentation, this treatment showed to have a negative impact towards the loss of ^{14}C-phenanthrene residues. Although no definitive cause for these findings was established, it may be related to the introduced degrader environmental adaptation and/or degradative competition between indigenous and inoculated degrader populations (Goldstein et al., 1985; Macleod and Semple, 2002; Mueller and Shann, 2007; Towell et al., 2011a). Additionally, this behaviour could be the consequence of a combined effect of the nutrient addition and soil pre-treatment (soil sieving), where the latter has already been observed to produce a disruption within the indigenous microbial populations, (Jiang et al., 2016).

4.2 ^{14}C-Phenanthrene and ^{14}C-octacosane degradation in soils

In this study, the indigenous microflora of both soils demonstrated high catabolic potential to degrade ^{14}C-phenanthene. The biodegradation of petroleum hydrocarbons is widely acknowledged to vary between different hydrocarbons, with physico-chemical properties such as molecular structure, size, hydrophobicity and solubility suggested to strongly influence their degradation potential (Riding et al., 2013; Yu et al., 2018). Moreover, the general increase of mineralisation of ^{14}C-phenanthene suggests that a s period of microbial growth and adaptation is required before extensive mineralisation (Macleod and Semple,

2002; Mueller and Shann, 2007; Vázquez-Cuevas et al., 2018). Overall, differences in initial ^{14}C-phenanthrene degradative activity are proposed to be related to the soils contamination history, and concentration of phenanthrene to which the indigenous microflora have been exposed (Spain and Van Veld, 1983; Grosser et al., 1995; Towell et al., 2011a).

In the case of octacosane, it has been pointed that the degradation and bioremediation potential of aliphatic hydrocarbons is often lower than aromatics (Chaíneau et al., 1995; Löser et al., 1999; Huesemann et al., 2003, 2004; Stroud et al., 2007). In specific, results showing a lower biodegradability of ^{14}C-octacosane when compared against ^{14}C-phenanthene may be attributed to the structural complexity, higher molecular weight and hydrophobicity of octacosane, limiting its biodegradability, mass transfer from the sorbed state, and/or increasing partitioning and retention into the soil matrix (Löser et al., 1999; Watts and Stanton, 1999; Reid et al., 2000; Huesemann et al., 2004).

Several studies have suggested that factors influencing contaminant bioaccessibility, such as SOM, may have more of an impact on aliphatic hydrocarbons, as opposed to aromatics (Löser et al., 1999; Watts and Stanton, 1999; Huesemann et al., 2003, 2004; Stroud et al., 2007). In specific, SOM is known to be a strong sorbent of organic contaminants, with the rate, strength and extent at which contaminants are retained influenced by their hydrophobicity (Reid et al., 2000; Huesemann et al., 2003; Semple et al., 2007). As significant differences in ^{14}C-hydrocarbon mineralisation between soils were only determined for ^{14}C-octacosane, these findings may be due to the increased sorption and retention of ^{14}C-octacosane in the soil with higher SOM/OC content, combined with the more hydrophobic nature of this hydrocarbon compared to phenanthrene (Löser et al., 1999; Reid et al., 2000; Huesemann et al., 2004). This would be expected to reduce the labile and rapidly desorbing octacosane fractions and subsequent degradation in this soil (Semple et al., 2007).

In addition to this, another important component to take into account are the inherent processes associated with the microbial activity that can lead to modifications of the biodegradation. In specific, the production of natural surfactants by some bacteria has been pointed to have contrasting effects towards microbial activity and ultimately reflecting on their usage of hydrocarbons as a carbon source. As a large number of microbial strains have been shown to modify their environment in order to increase their sources of carbon and nutrients. Authors have demonstrated that although rhamnolipids (a glycolipid produced by bacteria) are typically acknowledged to increase biodegradation of hydrocarbons, these can also represent a preferential source of carbon for bacteria (Akbari et al., 2021) and promote larger microbial populations within less diverse communities (Crampon et al., 2017). When combining these factors it can be identified that larger hydrocarbon degrading microbial numbers, as of the case of this study, are not necessarily going to reflect as a higher biodegradation. This is especially important when considering that *Pseudomonas* sp. is one of the most common hydrocarbon degrading bacteria with a well-established capacity to produce rhamnolipids (Akbari et al., 2021).

4.2.1 Biostimulation and bioaugmentation

The success of biostimulation, bioaugmentation and natural attenuation remediation schemes has been reported to vary widely for different hydrocarbons and soils (Jobson et al., 1974; Bento et al., 2005; Ramírez et al., 2009). Overall results from this study showed no impact of biostimulation or bioaugmentation towards the biodegradation of ^{14}C-phenanthrene. Comparable findings have been reported and ascribed to low nutrient availability, soil heterogeneity and the presence of an established indigenous degrader population (Bento et al., 2005; Towell et al., 2011a). Although in this study no definitive cause was established, it may be related to chemical reactions induced by the nutrient addition. This has been observed

to reflect as a range of modifications within the soil including acidification of the soil (Sarkar et al., 2005) and inhibition of the microbial degradation process due to overdose of nutrients, specially nitrogen forms (Ramadass et al., 2018). Moreover, this same behaviour at the latest stage (341 d) is also in agreement with other studies which have suggested that natural attenuation can be an efficient remediation method for petroleum-contaminated soils; and that bioaugmentation may not result in enhanced biodegradation performance and thus justify the remediation costs involved (Bento et al., 2005; Ramadass et al., 2018). Another important factor limiting the expected enhancing effects of nutrients towards biodegradation of hydrocarbons can be presumed to be linked to soil organic matter (SOM). In the case of this study SOM is significantly higher in soil B than A. As this particular trait has been acknowledged to inhibit mass transfer processes in soil due to sorption processes (Semple et al., 2003) , it is possible that the amended nutrients were not available for the bacterial consortium to use. This sportive behaviour might also be magnified by de presence of complex mixtures of contaminants present in crude oil, diesel and/or petrol, which are commonly processed and used in refinery facilities. Moreover, in addition to the influence of these fuels towards the sequestration of nutrients in soil, they can also contain other compounds that could negatively reflect on the biodegradation process due to acute toxicity (Benyahia et al., 2005; Vázquez-Cuevas et al., 2018). Acute toxic effects might be especially important in the case of soil B where there is no history of previous treatment. In this soil some of the semi volatile fractions of these complex mixtures might have gotten mobilised as a consequence of the manipulation of the soil and ultimately have a negative effect on the microbial metabolism.

4.3 Extractability of ^{14}C-phenanthrene and ^{14}C-octacosane associated activity from soils

Traditionally, contaminated land assessment, regulation and biological remediation clean up targets were quantified by total contaminant burdens in soil, with harsh and vigorous solvent extractions employed to determine total contaminant-soil concentrations. However, there has been a change in the way contaminant risk and bioremediation action is assessed, with significant emphasis now placed on contaminant bioaccessibility and its accurate measurement (Semple et al., 2004; Ortega-Calvo et al., 2015). In the case of the total extractable ^{14}C-phenanthrene and ^{14}C-octacosane, the consistent larger amounts of hydrocarbons extracted by DCM than by methanol:water and HPCD can be considered as an indication of low bioaccessibility (Ortega-Calvo et al., 2015). These findings are comparable with several other studies which demonstrate that this exhaustive extraction method overestimates bioaccessibility giving an inadequate representation of contaminant degradability in soils (Kelsey et al., 1997; Umeh et al., 2017; Anyanwu and Semple, 2018).

Contaminant sequestration is widely acknowledged to be governed by sorption into/onto OM/OC, with this considered to be the principal process in soils containing > 0.1% OC (Pignatello and Xing, 1996; Cornelissen et al., 1998; Nam et al., 1998; Lueking et al., 2000; Yang et al., 2010; Rhodes et al., 2012). Accordingly, it is probable that the fraction of non-extractable ^{14}C-hydrocarbon residues were higher in soil B due to increased hydrocarbon sequestration with aging, resulting from the higher SOM/OC of this soil. This may be attributed to the very hydrophobic nature of octacosane and a reduction in extraction efficiency due to the extremely high SOM content of the soil (Stroud et al., 2007).

Nutrient and bioaugmentation treatments had a varying impact on the DCM-extractability and aging of ^{14}C-octacosane in soils. This may be linked to high degradative activity resultant from the addition of catabolically active degraders, which caused an increase in the formation of non-extractable octacosane residues in this soil over time (Macleod and Semple, 2003;

Riding et al., 2013). Furthermore, correlations between decreasing contaminant bioaccessibility due to increased sorption in soils with higher SOM concentration and aging have been reported (Jones and Edwards, 1998; Nam et al., 1998; Yang et al., 2010; Luo et al., 2012).

Methanol:water and HPCD extractability was lower when evaluating ^{14}C-octacosene than in the case of ^{14}C-phenanthrene. Hydrophobicity is thought to significantly influence contaminant mobility and biological availability in soil, with hydrophobic contaminants typically observed to be more strongly sorbed to the soil matrix (Löser et al., 1999; Reid et al., 2000; Gunasekara and Xing, 2003). Due to octacosane having a more hydrophobic nature, it is plausible it may partition more strongly within the solid soil phases and thus be less readily extracted, compared to phenanthrene. Interestingly, as opposed to ^{14}C-phenanthrene, ^{14}C-octacosane extraction (by both methanol:water and HPCD) increased over the ageing period. This indicates an increase in bioaccessibility and possible contaminant remobilisation over the aging period (Riding et al., 2013). Due to the low amounts of ^{14}C-octacosane extracted at 0 and 31 d (for all soils and treatments), another plausible explanation is low methanol:water and HPCD extraction efficiency for this ^{14}C-hydrocarbon (Reid et al., 2000), as it will be further discussed.

4.3.1 Comparisons between methanol:water and HPCD-extractability and mineralisation of ^{14}C-phenanthrene and ^{14}C-octacosane in soils

There has been considerable research surrounding the development of less exhaustive extraction techniques to estimate the bioaccessibility and accurately predict the degradability of organic contaminants in soil (Reid et al., 2000; Stokes et al., 2005; Rhodes et al., 2008). However, it has also been observed that the ability of different extraction solutions to predict

bioaccessibility of the contaminant can show significant variations from case to case (Škulcová et al., 2016). Both methanol:water and HPCD extraction techniques appeared to be less predictive of ^{14}C-phenanthrene mineralisation for soil B. These findings are in contrast to several studies which have determined significant correlations between the total extents of ^{14}C-PAHs mineralised and extracted by HPCD (Patterson *et al.*, 2004; Semple *et al.*, 2006; Hofman *et al.*, 2008; Rhodes *et al.*, 2008b). Although, studies have reported that methanol:water extraction may underestimate the bioaccessibility and degradability of hydrophobic and low water soluble contaminants (Krauss et al., 2000; Macleod and Semple, 2000, 2003).

In this study, it is postulated that reductions in methanol:water and HPCD extraction efficiency due to the high levels of background hydrocarbon contamination (≥ 1.8% TPH) and/or the high OM concentrations (≥ 14%) of the soils may account for the considerably lower amounts of ^{14}C-phenanthrene extracted than mineralised (Reid et al., 2000; Stokes et al., 2005; Dandie et al., 2010). As for ^{14}C-octacosane, the absence of a clear and strong correlation might be due to steric constraints resulting from the higher molecular weight of octacosane and/or physico-chemical properties (high hydrophobicity, K_{ow} and low solubility) restricting transfer to the aqueous phase (Reid et al., 2000; Stokes et al., 2005; Papadopoulos et al., 2007; Stroud et al., 2008). Conversely, at 124 and 341 d methanol:water extraction generally overestimated the mineralisation of ^{14}C-octacosane, with extraction:mineralisation ratios consistently larger than 1 for control and treated soils.

5. Conclusion

Results showed that the degree to which hydrocarbons are degraded and/or retained in soils is strongly influenced by physical and chemical properties of the organic contaminant, SOM,

soil-contaminant contact time and the presence and activity of degrading microorganisms. In this study two contrasting hydrocarbons were analysed. As such, the PAH (phenanthrene) and the long-chained aliphatic hydrocarbon (octacosane) exhibited contrasting behaviours. Due to its extremely hydrophobic nature, octacosane showed to be more influenced by SOM/OC, having a higher inhibitory impact upon the bioavailability/bioaccessibility and its microbial degradation in soils. Results from this study clearly show that microbial degradation should not be considered as a cost-effective remediation approach without a more comprehensive evaluation of the partitioning, extractability and bioaccessibility of hydrocarbons in soil. Contrary to what is typically done, determining the likelihood of effective biodegradation by the stimulation of indigenous microorganisms or through bioaugmentation needs to be assessed by both chemical and biological measurements of bioaccessibility, rather than just by which is totally extractable from soil. However, specific characteristics of the soil must be considered to select the proper methods as in the case of soils with high loadings of organic matter and/or organic contaminants which might prevent accurate assessment of contaminant bioaccessibility, as measured by HPCD. Considering this, it is proposed that a combined and integrated biological/chemical approach is required to fully assess the bioremediation potential and thus determine suitable clean up targets for petroleum-contaminated sites.

Acknowledgments

The authors would like to thank the Engineering and Physical Sciences Research Council (EPSRC) and National Grid for funding this study. The project was also supported by the LINK Bioremediation programme (BIOREM_35), the Environment Agency of England and Wales (EA) and the Biotechnology and Biological Sciences Research Council BBSRC (Grant BB/B512432/1). GMVC would like to also thank the Mexican National Council for Science and Technology (CONACyT) for their financial support through a scholarship (No. 313807) to pursue doctoral studies at the Lancaster Environment Centre, Lancaster University.

References

Adedigba, B.M., Ogbonnaya, U.O., Vázquez-Cuevas, G.M., Semple, K.T., 2018. Optimisation of XAD extraction methodology for the assessment of biodegradation potential of 14C-phenanthrene in soil. Environ. Technol. Innov. 9, 140–150. https://doi.org/10.1016/j.eti.2017.11.011

Akbari, A., Kasprzyk, A., Galvez, R., Ghoshal, S., 2021. A rhamnolipid biosurfactant increased bacterial population size but hindered hydrocarbon biodegradation in weathered contaminated soils. Sci. Total Environ. In Press. https://doi.org/10.1016/j.scitotenv.2021.145441

Alexander, M., 2000. Aging, bioavaiability, and overestimation of risk from environmental pollutants. Environ. Sci. Technol. 34, 4259–4265. https://doi.org/10.1021/es001069+

Anyanwu, I.N., Semple, K.T., 2018. Impact of single and binary mixtures of phenanthrene and N-PAHs on microbial utilization of 14C-glucose in soil. Soil Biol. Biochem. 120, 222–229. https://doi.org/10.1016/j.soilbio.2018.02.009

Babu, A.G., Reja, S.I., Akhtar, N., Sultana, M., Deore, P.S., Ali, F.I., 2019. Bioremediation of Polycyclic Aromatic Hydrocarbons (PAHs): Current Practices and Outlook, in: Microbial Metabolism of Xenobiotic Compounds. Springer, pp. 189–216.

Bento, F.M., Camargo, F.A.O., Okeke, B.C., Frankenberger, W.T., 2005. Comparative bioremediation of soils contaminated with diesel oil by natural attenuation, biostimulation and bioaugmentation. Bioresour. Technol. 96, 1049–1055. https://doi.org/10.1016/j.biortech.2004.09.008

Benyahia, F., Abdulkarim, M., Zekri, A., Chaalal, O., Hasanain, H., 2005. Bioremediation of crude oil contaminated soils a black art or an engineering challenge? Process Saf. Environ. Prot. 83, 364–370. https://doi.org/10.1205/psep.04388

Chaíneau, C.H., Morel, J.L., Oudot, J., 1995. Microbial Degradation in Soil Microcosms of Fuel Oil Hydrocarbons from Drilling Cuttings. Environ. Sci. Technol. 29, 1615–1621. https://doi.org/10.1021/es00006a027

Chen, M., Xu, P., Zeng, G., Yang, C., Huang, D., Zhang, J., 2015. Bioremediation of soils contaminated with polycyclic aromatic hydrocarbons, petroleum, pesticides, chlorophenols and heavy metals by composting: Applications, microbes and future research needs. Biotechnol. Adv. https://doi.org/10.1016/j.biotechadv.2015.05.003

Cipullo, S., Prpich, G., Campo, P., Coulon, F., 2018. Assessing bioavailability of complex chemical mixtures in contaminated soils: Progress made and research needs. Sci. Total Environ. 615, 708–723. https://doi.org/10.1016/j.scitotenv.2017.09.321

Cornelissen, G., Rigterink, H., Ferdinandy, M.M.A., Van Noort, P.C.M., 1998. Rapidly desorbing fractions of PAHs in contaminated sediments as a predictor of the extent of bioremediation. Environ. Sci. Technol. 32, 966–970. https://doi.org/10.1021/es9704038

Crampon, M., Cébron, A., Portet-Koltalo, F., Uroz, S., Le Derf, F., Bodilis, J., 2017. Low effect of phenanthrene bioaccessibility on its biodegradation in diffusely contaminated soil. Environ. Pollut. 225, 663–673. https://doi.org/10.1016/j.envpol.2017.03.053

Dandie, C.E., Weber, J., Aleer, S., Adetutu, E.M., Ball, A.S., Juhasz, A.L., 2010. Assessment of five bioaccessibility assays for predicting the efficacy of petroleum hydrocarbon biodegradation in aged contaminated soils. Chemosphere 81, 1061–1068. https://doi.org/10.1016/j.chemosphere.2010.09.059

Dew, N.M., Paton, G.I., Semple, K.T., 2005. Prediction of [3-14C]phenyldodecane biodegradation in cable insulating oil-spiked soil using selected extraction techniques. Environ. Pollut. 138, 316–323. https://doi.org/10.1016/j.envpol.2005.03.009

Doick, K.J., Lee, P.H., Semple, K.T., 2003. Assessment of spiking procedures for the

introduction of a phenanthrene-LNAPL mixture into field-wet soil. Environ. Pollut. 126, 399–406. https://doi.org/10.1016/S0269-7491(03)00230-6

Duan, L., Naidu, R., Liu, Y., Palanisami, T., Dong, Z., Mallavarapu, M., Semple, K.T., 2015a. Effect of ageing on benzo[a]pyrene extractability in contrasting soils. J. Hazard. Mater. 296, 175–184. https://doi.org/10.1016/j.jhazmat.2015.04.050

Duan, L., Naidu, R., Thavamani, P., Meaklim, J., Megharaj, M., 2015b. Managing long-term polycyclic aromatic hydrocarbon contaminated soils: a risk-based approach. Environ. Sci. Pollut. Res. 22, 8927–8941. https://doi.org/10.1007/s11356-013-2270-0

Goldstein, R.M., Mallory, L.M., Alexander, M., 1985. Reasons for possible failure of inoculation to enhance biodegradation. Appl. Environ. Microbiol. 50, 977–983.

Grosser, R.J., Vestal, J.R., Warshawsky, D., 1995. Mineralization of polycyclic and N-heterocyclic aromatic compounds in hydrocarbon-contaminated soils. Environ. Toxicol. Chem. 14, 375–382. https://doi.org/10.1002/etc.5620140304

Gunasekara, A.S., Xing, B., 2003. Sorption and desorption of naphthalene by soil organic matter: importance of aromatic and aliphatic components. J. Environ. Qual. 32, 240–246. https://doi.org/10.2134/jeq2003.2400

Hofman, J., Rhodes, A., Semple, K.T., 2008. Fate and behaviour of phenanthrene in the natural and artificial soils. Environ. Pollut. 152, 468–475. https://doi.org/10.1016/j.envpol.2007.05.034

Huesemann, M.H., Hausmann, T.S., Fortman, T.J., 2004. Does bioavailability limit biodegradation ? A comparison of hydrocarbon biodegradation and desorption rates in aged soils. Biodegradation 15, 261–274. https://doi.org/10.1023/B:BIOD.0000042996.03551.f4

Huesemann, M.H., Hausmann, T.S., Fortman, T.J., 2003. Assessment of bioavailability

limitations during slurry biodegradation of petroleum hydrocarbons in aged soils. Environ. Toxicol. Chem. 22, 2853–2860. https://doi.org/10.1897/02-611

Jiang, Y., Brassington, K.J., Prpich, G., Paton, G.I., Semple, K.T., Pollard, S.J.T., Coulon, F., 2016. Insights into the biodegradation of weathered hydrocarbons in contaminated soils by bioaugmentation and nutrient stimulation. Chemosphere 161, 300–307. https://doi.org/10.1016/j.chemosphere.2016.07.032

Jobson, A., McLaughlin, M., Cook, F.D., Westlake, D.W., 1974. Effect of amendments on the microbial utilization of oil applied to soil. Appl. Microbiol. 27, 166–171.

Jones, D.L., Edwards, A.C., 1998. Influence of sorption on the biological utilization of two simple carbon substrates. Soil Biol. Biochem. 30, 1895–1902. https://doi.org/10.1016/S0038-0717(98)00060-1

Kelsey, J.W., Kottler, B.D., Alexander, M., 1997. Selective chemiscal extractants to predict bioavaiability of soil-aged organic chemicals. Environ. Sci. Technol. 31, 214–217.

Krauss, M., Wilcke, W., Zech, W., 2000. Availability of polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) to earthworms in urban soils. Environ. Sci. Technol. 34, 4335–4340. https://doi.org/10.1021/es001137s

Leahy, J.G., Colwell, R.R., 1990. Microbial degradation of hydrocarbons in the environment. Microbiol. Rev. 54, 305–315. https://doi.org/

Löser, C., Seidel, H., Hoffmann, P., Zehnsdorf, A., 1999. Bioavailability of hydrocarbons during microbial remediation of a sandy soil. Appl. Microbiol. Biotechnol. 51, 105–111. https://doi.org/10.1007/s002530051370

Lueking, A.D., Huang, W.L., Soderstrom-Schwarz, S., Kim, M.S., Weber, W.J., 2000. Relationship of soil organic matter characteristics to organic contaminant sequestration and bioavailability. J. Environ. Qual. 29, 317–323.

Luo, L., Lin, S., Huang, H., Zhang, S., 2012. Relationships between aging of PAHs and soil properties. Environ. Pollut. 170, 177–182. https://doi.org/10.1016/j.envpol.2012.07.003

Macleod, C.J.A., Semple, K.T., 2003. Sequential extraction of low concentrations of pyrene and formation of non-extractable residues in sterile and non-sterile soils. Soil Biol. Biochem. 35, 1443–1450. https://doi.org/10.1016/S0038-0717(03)00238-4

Macleod, C.J.A., Semple, K.T., 2002. The adaptation of two similar soils to pyrene catabolism. Environ. Pollut. 119, 357–364. https://doi.org/10.1016/S0269-7491(01)00343-8

Macleod, C.J.A., Semple, K.T., 2000. Influence of contact time on extractability and degradation of pyrene in soils. Environ. Sci. Technol. 34, 4952–4957. https://doi.org/10.1021/es000061x

Masy, T., Demanèche, S., Tromme, O., Thonart, P., Jacques, P., Hiligsmann, S., Vogel, T.M., 2016. Hydrocarbon biostimulation and bioaugmentation in organic carbon and clay-rich soils. Soil Biol. Biochem. 99, 66–74. https://doi.org/10.1016/j.soilbio.2016.04.016

Mueller, K.E., Shann, J.R., 2007. Effects of tree root-derived substrates and inorganic nutrients on pyrene mineralization in rhizosphere and bulk soil. J. Environ. Qual. 36, 120. https://doi.org/10.2134/jeq2006.0130

Nam, K., Chung, N., Alexander, M., 1998. Relationship between organic matter content of soil and the sequestration of phenanthrene. Environ. Sci. Technol. 32, 3785–3788. https://doi.org/10.1021/es980428m

Okere, U. V., Cabrerizo, A., Dachs, J., Ogbonnaya, U.O., Jones, K.C., Semple, K.T., 2017. Effects of pre-exposure on the indigenous biodegradation of14C-phenanthrene in Antarctic soils. Int. Biodeterior. Biodegrad. 125, 189–199. https://doi.org/10.1016/j.ibiod.2017.09.013

Ortega-Calvo, J.J., Harmsen, J., Parsons, J.R., Semple, K.T., Aitken, M.D., Ajao, C., Eadsforth, C., Galay-Burgos, M., Naidu, R., Oliver, R., Peijnenburg, W.J.G.M., Römbke, J., Streck, G., Versonnen, B., 2015. From bioavailability science to regulation of organic chemicals. Environ. Sci. Technol. 49, 10255–10264. https://doi.org/10.1021/acs.est.5b02412

Ortega-Calvo, J.J., Tejeda-Agredano, M.C., Jimenez-Sanchez, C., Congiu, E., Sungthong, R., Niqui-Arroyo, J.L., Cantos, M., 2013. Is it possible to increase bioavailability but not environmental risk of PAHs in bioremediation? J. Hazard. Mater. 261, 733–745. https://doi.org/10.1016/j.jhazmat.2013.03.042

Papadopoulos, A., Paton, G.I., Reid, B.J., Semple, K.T., 2007. Prediction of PAH biodegradation in field contaminated soils using a cyclodextrin extraction technique. J. Environ. Monit. 9, 516. https://doi.org/10.1039/b700720e

Patterson, C.J., Semple, K.T., Paton, G.I., 2004. Non-exhaustive extraction techniques (NEETs) for the prediction of naphthalene mineralisation in soil. FEMS Microbiol. Lett. 241, 215–220. https://doi.org/10.1016/j.femsle.2004.10.023

Pignatello, J.J., Xing, B., 1996. Mechanisms of slow sorption of organic chemicals to natural particles. Environ. Sci. Technol. 30, 1–11. https://doi.org/10.1021/es940683g

Ramadass, K., Megharaj, M., Venkateswarlu, K., Naidu, R., 2018. Bioavailability of weathered hydrocarbons in engine oil-contaminated soil: Impact of bioaugmentation mediated by Pseudomonas spp. on bioremediation. Sci. Total Environ. 636, 968–974. https://doi.org/10.1016/j.scitotenv.2018.04.379

Ramírez, M.E., Zapién, B., Zegarra, H.G., Rojas, N.G., Fernández, L.C., 2009. Assessment of hydrocarbon biodegradability in clayed and weathered polluted soils. Int. Biodeterior. Biodegrad. 63, 347–353. https://doi.org/10.1016/j.ibiod.2008.11.010

Reid, B.J., Jones, K.C., Semple, K.T., 2000. Bioavailability of persistent organic pollutants in soils and sediments—a perspective on mechanisms, consequences and assessment. Environ. Pollut. 108, 103–112. https://doi.org/10.1016/S0269-7491(99)00206-7

Reid, B.J., MacLeod, C.J.A., Lee, P.H., Morriss, A.W.J., Stokes, J.D., Semple, K.T., 2001. A simple 14C-respirometric method for assessing microbial catabolic potential and contaminant bioavailability. FEMS Microbiol. Lett. 196, 141–146. https://doi.org/10.1016/S0378-1097(01)00062-3

Reid, Brian J., Stokes, J.D., Jones, K.C., Semple, K.T., 2000. Nonexhaustive cyclodextrin-based extraction technique for the evaluation of PAH bioavailability. Environ. Sci. Technol. 34, 3174–3179. https://doi.org/10.1021/es990946c

Rhodes, A.H., Dew, N.M., Semple, K.T., 2008. Relationship between cyclodextrin extraction and biodegradation of phenanthrene in soil. Environ. Toxicol. Chem. 27, 1488–95. https://doi.org/10.1897/07-363

Rhodes, A.H., McAllister, L.E., Chen, R., Semple, K.T., 2010a. Impact of activated charcoal on the mineralisation of 14C-phenanthrene in soils. Chemosphere 79, 463–469. https://doi.org/10.1016/j.chemosphere.2010.01.032

Rhodes, A.H., McAllister, L.E., Semple, K.T., 2010b. Linking desorption kinetics to phenanthrene biodegradation in soil. Environ. Pollut. 158, 1348–1353. https://doi.org/10.1016/j.envpol.2010.01.008

Rhodes, A.H., Riding, M.J., McAllister, L.E., Lee, K., Semple, K.T., 2012. Influence of activated charcoal on desorption kinetics and biodegradation of phenanthrene in soil. Environ. Sci. Technol. 46, 12445–12451. https://doi.org/10.1021/es3025098

Riding, M.J., Doick, K.J., Martin, F.L., Jones, K.C., Semple, K.T., 2013. Chemical measures of bioavailability/bioaccessibility of PAHs in soil: Fundamentals to application. J.

Hazard. Mater. 261, 687–700. https://doi.org/10.1016/j.jhazmat.2013.03.033

Risdon, G.C., Pollard, S.J.T., Brassington, K.J., McEwan, J.N., Paton, G.I., Semple, K.T., Coulon, F., 2008. Development of an analytical procedure for weathered hydrocarbon contaminated soils within a UK risk-based framework. Anal. Chem. 80, 7090–7096. https://doi.org/10.1021/ac800698g

Samanta, S.K., Singh, O. V, Jain, R.K., 2002. Polycyclic aromatic hydrocarbons : environmental pollution and bioremediation. Trends Biotechnol. 20, 243–248. https://doi.org/10.1016/S0167-7799(02)01943-1

Sarkar, D., Ferguson, M., Datta, R., Birnbaum, S., 2005. Bioremediation of petroleum hydrocarbons in contaminated soils: Comparison of biosolids addition, carbon supplementation, and monitored natural attenuation. Environ. Pollut. 136, 187–195. https://doi.org/10.1016/j.envpol.2004.09.025

Semple, K.T., Dew, N.M., Doick, K.J., Rhodes, A.H., 2006. Can microbial mineralization be used to estimate microbial availability of organic contaminants in soil? Environ. Pollut. 140, 164–172. https://doi.org/10.1016/j.envpol.2005.06.009

Semple, K.T., Doick, K.J., Jones, K.C., Burauel, P., Craven, A., Harms, H., 2004. Defining bioavailability and bioaccessibility of contaminated soil and sediment is complicated. Environ. Sci. Technol. 38, 228A-231A. https://doi.org/10.1021/es040548w

Semple, K.T., Doick, K.J., Wick, L.Y., Harms, H., 2007. Microbial interactions with organic contaminants in soil: Definitions, processes and measurement. Environ. Pollut. 150, 166–176. https://doi.org/10.1016/j.envpol.2007.07.023

Semple, K.T., Morriss, a. W.J., Paton, G.I., 2003. Bioavailability of hydrophobic organic contaminants in soils: fundamental concepts and techniques for analysis. Eur. J. Soil Sci. 54, 809–818. https://doi.org/10.1046/j.1365-2389.2003.00564.x

Semple, K.T., Riding, M.J., McAllister, L.E., Sopena-Vazquez, F., Bending, G.D., 2013. Impact of black carbon on the bioaccessibility of organic contaminants in soil. J. Hazard. Mater. 261, 808–816. https://doi.org/10.1016/j.jhazmat.2013.03.032

Škulcová, L., Neuwirthová, N., Hofman, J., Bielská, L., 2016. Assessment of the biological and chemical availability of the freshly spiked and aged DDE in soil. Environ. Pollut. 212, 105–112. https://doi.org/10.1016/j.envpol.2016.01.042

Spain, J.C., Van Veld, P.A., 1983. Adaptation of natural microbial communities to degradation of xenobiotic compounds: Effects of concentration, exposure time, inoculum, and chemical structure. Appl. Environ. Microbiol. 45, 428–435.

Stokes, J.D., Paton, G.I., Semple, K.T., 2005. Behaviour and assessment of bioavailability of organic contaminants in soil: relevance for risk assessment and remediation. Soil Use Manag. 21, 475–486. https://doi.org/10.1079/SUM2005347

Stokes, Joanna D, Wilkinson, A., Reid, B.J., Jones, K.C., Semple, K.T., 2005. Prediction of polycyclic aromatic hydrocarbon biodegradation in contaminated soils using an aqueous hydroxypropyl-β-cyclodextrin extraction technique. Environ. Toxicol. Chem. 24, 1325. https://doi.org/10.1897/04-336r.1

Stroud, J.L., Paton, G.I., Semple, K.T., 2008. Linking chemical extraction to microbial degradation of 14C-hexadecane in soil. Environ. Pollut. 156, 474–481. https://doi.org/10.1016/j.envpol.2008.01.018

Stroud, Jacqueline L., Paton, G.I., Semple, K.T., 2007. Importance of chemical structure on the development of hydrocarbon catabolism in soil. FEMS Microbiol. Lett. 272, 120–126. https://doi.org/10.1111/j.1574-6968.2007.00750.x

Stroud, J. L., Paton, G.I., Semple, K.T., 2007. Microbe-aliphatic hydrocarbon interactions in soil: Implications for biodegradation and bioremediation. J. Appl. Microbiol. 102, 1239–

1253. https://doi.org/10.1111/j.1365-2672.2007.03401.x

Swindell, A.L., Reid, B.J., 2006. Influence of diesel concentration on the fate of phenanthrene in soil. Environ. Pollut. 140, 79–86. https://doi.org/10.1016/j.envpol.2005.06.022

Towell, M.G., Bellarby, J., Paton, G.I., Coulon, F., Pollard, S.J.T., Semple, K.T., 2011a. Mineralisation of target hydrocarbons in three contaminated soils from former refinery facilities. Environ. Pollut. 159, 515–523. https://doi.org/10.1016/j.envpol.2010.10.015

Towell, M.G., Paton, G.I., Semple, K.T., 2011b. The biodegradation of cable oil components: Impact of oil concentration, nutrient addition and bioaugmentation. Environ. Pollut. 159, 3777–3783. https://doi.org/10.1016/j.envpol.2011.06.043

Tripathi, V., Fraceto, L.F., Abhilash, P.C., 2015. Sustainable clean-up technologies for soils contaminated with multiple pollutants: Plant-microbe-pollutant and climate nexus. Ecol. Eng. 82, 330–335. https://doi.org/10.1016/j.ecoleng.2015.05.027

Umeh, A.C., Duan, L., Naidu, R., Semple, K.T., 2017. Residual hydrophobic organic contaminants in soil: Are they a barrier to risk-based approaches for managing contaminated land? Environ. Int. 98, 18–34. https://doi.org/10.1016/j.envint.2016.09.025

Varjani, S.J., 2017. Microbial degradation of petroleum hydrocarbons. Bioresour. Technol. https://doi.org/10.1016/j.biortech.2016.10.037

Varjani, S.J., Upasani, V.N., 2017. A new look on factors affecting microbial degradation of petroleum hydrocarbon pollutants. Int. Biodeterior. Biodegrad. https://doi.org/10.1016/j.ibiod.2017.02.006

Vázquez-Cuevas, G.M., Semple, K.T., 2016. Measurement of Hydrocarbon Bioavailability in Soil, in: McGenity, T., Timmis, K., Nogales, B. (Eds.), Hydrocarbon and Lipid Microbiology Protocols - Springer Protocols Handbooks. Springer, Berlin, Heidelberg,

pp. 231–246. https://doi.org/10.1007/8623_2016_216

Vázquez-Cuevas, G.M., Stevens, C.J., Semple, K.T., 2018. Enhancement of14C-phenanthrene mineralisation in the presence of plant-root biomass in PAH-NAPL amended soil. Int. Biodeterior. Biodegrad. 126, 78–85. https://doi.org/10.1016/j.ibiod.2017.09.021

Wang, C., Sun, H., Liu, H., Wang, B., 2014. Biodegradation of pyrene by Phanerochaete chrysosporium and enzyme activities in soils: Effect of SOM, sterilization and aging. J. Environ. Sci. (China) 26, 1135–1144. https://doi.org/10.1016/S1001-0742(13)60507-0

Watts, R.J., Stanton, P.C., 1999. Mineralization of sorbed and NAPL-phase hexadecane by catalyzed hydrogen peroxide. Water Res. 33, 1405–1414. https://doi.org/10.1016/S0043-1354(98)00343-1

Yang, Y., Zhang, N., Xue, M., Tao, S., 2010. Impact of soil organic matter on the distribution of polycyclic aromatic hydrocarbons (PAHs) in soils. Environ. Pollut. 158, 2170–2174. https://doi.org/10.1016/j.envpol.2010.02.019

Yu, L., Duan, L., Naidu, R., Semple, K.T., 2018. Abiotic factors controlling bioavailability and bioaccessibility of polycyclic aromatic hydrocarbons in soil: Putting together a bigger picture. Sci. Total Environ. 613–614, 1140–1153. https://doi.org/10.1016/j.scitotenv.2017.09.025

Yu, L., Vázquez-Cuevas, G., Duan, L., Semple, K.T., 2016. Buffered cyclodextrin extraction of14C-phenanthrene from black carbon amended soil. Environ. Technol. Innov. 6, 177–184. https://doi.org/10.1016/j.eti.2016.09.002

Zhen, L., Hu, T., Lv, R., Wu, Y., Chang, F., Jia, F., Gu, J., 2021. Succession of microbial communities and synergetic effects during bioremediation of petroleum hydrocarbon-contaminated soil enhanced by chemical oxidation. J. Hazard. Mater. 410, 124869.

https://doi.org/10.1016/j.jhazmat.2020.124869

Table 2. Total amounts of ^{14}C-phenanthrene associated activity remaining and extracted by DCM, methanol:water and HPCD for soil A and B treatments at the beginning of the investigation (0 d) and after 31, 62, 124 and 341 d incubation. Values represent the mean ± SEM (*n* = 3).

Aging Period (days)	Soil Treatment	Soil A				Soil B			
		Total ^{14}C in soil (%)	DCM extracted (%)	Methanol:water extracted (%)	HPCD extracted (%)	Total ^{14}C in soil (%)	DCM extracted (%)	Methanol:water extracted (%)	HPCD extracted (%)
0	Control	100 ± 0.00	95.82 ± 2.59	54.71 ± 2.02	55.95 ± 2.30	100 ± 0.00	91.27 ± 0.86	43.64 ± 0.86	52.40 ± 0.40
	Nutrient	100 ± 0.00	92.81 ± 4.37	54.40 ± 3.04	51.55 ± 2.74	100 ± 0.00	94.02 ± 2.97	38.90 ± 1.86	55.10 ± 1.76
	Bioaugmented	100 ± 0.00	93.03 ± 1.41	56.27 ± 0.72	60.52 ± 2.04	100 ± 0.00	96.29 ± 1.25	46.95 ± 1.27	46.89 ± 0.30
31	Control	98.20 ± 0.85	94.37 ± 4.64	52.43 ± 1.82	46.16 ± 1.62	89.65 ± 2.80	90.26 ± 0.76	42.87 ± 2.14	43.38 ± 3.86
	Nutrient	60.41 ± 7.21	92.87 ± 0.48	48.25 ± 1.95	47.64 ± 1.81	86.69 ± 0.49	91.71 ± 3.15	39.98 ± 1.76	38.93 ± 2.52
	Bioaugmented	88.37 ± 4.65	80.34 ± 5.34	59.88 ± 1.85	60.01 ± 1.58	86.62 ± 3.96	89.14 ± 4.61	40.37 ± 1.61	37.96 ± 1.83
62	Control	22.95 ± 0.66	87.50 ± 1.81	42.93 ± 1.84	29.92 ± 1.61	49.93 ± 3.25	87.01 ± 3.55	28.29 ± 2.12	23.80 ± 0.94
	Nutrient	49.42 ± 0.14	87.01 ± 3.55	44.52 ± 0.95	38.22 ± 1.31	45.50 ± 2.45	91.19 ± 0.85	26.61 ± 1.91	28.39 ± 3.38
	Bioaugmented	62.46 ± 0.28	80.40 ± 0.87	25.09 ± 1.64	58.31 ± 0.55	65.65 ± 0.27	88.79 ± 3.51	37.59 ± 1.45	21.56 ± 1.31
124	Control	22.63 ± 1.04	83.26 ± 0.91	45.76 ± 3.04	30.52 ± 0.62	46.40 ± 1.74	75.16 ± 0.58	24.55 ± 2.56	20.08 ± 1.53
	Nutrient	34.63 ± 0.34	85.22 ± 0.51	41.11 ± 0.61	28.71 ± 1.23	34.82 ± 0.77	81.34 ± 0.16	16.80 ± 1.03	24.92 ± 2.08
	Bioaugmented	49.71 ± 1.17	77.50 ± 1.35	20.95 ± 0.94	55.90 ± 1.51	52.19 ± 3.69	77.09 ± 2.36	21.26 ± 1.62	16.15 ± 0.80
341	Control	21.20 ± 0.21	81.75 ± 1.35	38.66 ± 1.03	24.22 ± 0.82	45.05 ± 1.92	71.05 ± 0.51	25.08 ± 2.87	14.99 ± 1.07
	Nutrient	31.06 ± 1.54	81.57 ± 1.86	33.75 ± 1.16	26.01 ± 1.20	33.44 ± 1.09	72.44 ± 0.78	24.03 ± 1.42	14.86 ± 0.41
	Bioaugmented	48.75 ± 1.90	80.17 ± 1.61	19.65 ± 0.79	50.80 ± 1.19	48.92 ± 2.54	73.56 ± 1.70	23.42 ± 1.04	14.30 ± 1.62

Table 3. Total amounts of ^{14}C-octacosane associated activity remaining and extracted by DCM, methanol:water and HPCD for soil A and B treatments at the beginning of the investigation (0 d) and after 31, 62, 124 and 341 d incubation. Values represent the mean ± SEM (n = 3).

Aging Period (days)	Soil Treatment	Soil A				Soil B			
		Total ^{14}C in soil (%)	DCM extracted (%)	Methanol:water extracted (%)	HPCD extracted (%)	Total ^{14}C in soil (%)	DCM extracted (%)	Methanol:water extracted (%)	HPCD extracted (%)
0	Control	100 ± 0.00	95.10 ± 1.56	2.26 ± 0.17	3.91 ± 0.29	100 ± 0.00	53.97 ± 3.33	1.55 ± 0.39	5.27 ± 0.67
	Nutrient	100 ± 0.00	98.82 ± 1.45	1.52 ± 0.11	6.38 ± 0.70	100 ± 0.00	52.51 ± 3.99	1.94 ± 0.43	4.91 ± 0.45
	Bioaugmented	100 ± 0.00	98.07 ± 0.49	1.34 ± 0.19	4.55 ± 0.56	100 ± 0.00	50.64 ± 2.87	2.82 ± 0.08	5.10 ± 0.40
31	Control	73.72 ± 5.32	93.84 ± 2.71	3.58± 0.32	9.89 ± 0.64	74.15 ± 4.77	43.49 ± 0.77	3.59 ± 0.52	10.10 ± 0.77
	Nutrient	91.62 ± 5.78	94.89 ± 3.24	3.60 ± 0.47	9.18 ± 0.75	51.57 ± 4.80	53.79 ± 1.70	5.38 ± 0.59	11.85 ± 0.15
	Bioaugmented	63.64 ± 3.73	82.38 ± 1.41	2.00 ± 0.38	5.25 ± 0.54	88.64 ± 0.46	45.26 ± 2.17	2.87 ± 0.28	5.84 ± 0.24
62	Control	65.11 ± 0.34	91.59 ± 4.38	17.90 ± 0.70	12.74 ± 0.24	64.78 ± 3.64	36.64 ± 1.28	11.76 ± 1.01	11.88 ± 0.86
	Nutrient	79.02 ± 1.88	93.90 ± 4.41	30.00 ± 1.33	13.17 ± 0.39	37.74 ± 0.85	45.14 ± 2.86	8.26 ± 0.91	11.28 ± 0.15
	Bioaugmented	12.28 ± 1.94	77.74 ± 2.74	37.45 ± 2.67	15.01 ± 0.38	30.10 ± 2.95	39.22 ± 3.87	16.31 ± 0.79	17.85 ± 0.42
124	Control	43.69 ± 3.36	91.63 ± 2.94	24.72 ± 1.10	13.67 ± 0.57	51.86 ± 3.36	27.36 ± 2.19	15.56 ± 0.08	17.79 ± 0.52
	Nutrient	54.41 ± 2.41	90.34 ± 6.90	30.99 ± 2.81	13.58 ± 0.37	30.03 ± 0.94	38.03 ± 1.51	16.30 ± 0.68	12.64 ± 0.69
	Bioaugmented	5.86 ± 1.09	78.21 ± 3.63	37.15 ± 1.76	15.20 ± 0.62	27.80 ± 2.66	32.99 ± 0.94	20.65 ± 0.66	20.75 ± 0.75
341	Control	37.47 ± 0.52	89.64 ± 1.07	25.91 ± 1.82	14.55 ± 0.69	38.21 ± 0.84	24.35 ± 0.79	20.82 ± 1.29	15.15 ± 0.52
	Nutrient	52.83 ± 2.50	91.63 ± 2.47	47.79 ± 0.70	16.22 ± 0.55	28.62 ± 1.00	36.26 ± 1.77	17.84 ± 0.56	14.75 ± 0.33
	Bioaugmented	4.06 ± 0.56	74.18 ± 1.54	41.20 ± 2.75	17.81 ± 0.19	23.84 ± 1.35	29.91 ± 2.90	21.26 ± 0.91	20.45 ± 0.42

Table 4. Total extents, maximum rates and lag times of ^{14}C-phenanthrene mineralisation for soil A and B treatments at each timepoint over 341 d. Values represent the mean ± SEM (n = 3).

Aging Period (days)	Soil Treatment	Soil A			Soil B		
		Total extent (%)	Maximum rate (% h^{-1})	Lag time (h)	Total extent (%)	Maximum rate (% h^{-1})	Lag time (h)
0	Control	78.88 ± 1.29	1.67 ± 0.04	19.59 ± 0.26	70.24 ± 1.07	1.00 ± 0.00	42.14 ± 0.83
	Nutrient	77.46 ± 0.48	1.63 ± 0.04	19.98 ± 0.78	74.15 ± 0.44	1.15 ± 0.03	27.21 ± 0.99
	Bioaugmented	80.58 ± 1.01	1.75 ± 0.02	20.37 ± 0.42	72.54 ± 1.46	1.25 ± 0.11	27.00 ± 0.37
31	Control	62.23 ± 1.01	0.46 ± 0.00	23.42 ± 1.05	63.92 ± 0.10	0.43 ± 0.01	48.04 ± 5.13
	Nutrient	60.35 ± 0.94	0.47 ± 0.01	20.96 ± 0.69	48.78 ± 0.82	0.36 ± 0.01	50.71 ± 1.31
	Bioaugmented	61.99 ± 1.26	0.50 ± 0.03	25.43 ± 0.53	60.55 ± 0.86	0.39 ± 0.01	38.78 ± 3.80
62	Control	61.08 ± 1.65	0.39 ± 0.01	22.20 ± 1.06	63.33 ± 1.63	0.44 ± 0.03	34.37 ± 0.17
	Nutrient	51.66 ± 0.11	0.30 ± 0.03	23.53 ± 0.69	11.04 ± 0.59	0.07 ± 0.00	92.12 ± 4.50
	Bioaugmented	65.08 ± 1.38	0.40 ± 0.02	22.88 ± 2.56	67.92 ± 1.10	0.43 ± 0.01	33.30 ± 0.47
124	Control	48.19 ± 2.02	0.49 ± 0.02	25.66 ± 1.74	45.32 ± 1.42	0.47 ± 0.03	28.20 ± 1.58
	Nutrient	52.19 ± 1.78	0.52 ± 0.04	22.77 ± 1.41	11.39 ± 0.20	0.06 ± 0.01	96.37 ± 5.10
	Bioaugmented	52.88 ± 1.02	0.50 ± 0.03	27.37 ± 1.56	53.65 ± 2.24	0.51 ± 0.04	34.70 ± 1.27
341	Control	45.59 ± 1.08	0.40 ± 0.03	35.50 ± 0.79	47.64 ± 0.80	0.45 ± 0.02	36.02 ± 0.86
	Nutrient	47.17 ± 0.99	0.42 ± 0.02	29.85 ± 0.88	10.22 ± 0.24	0.05 ± 0.00	95.69 ± 1.02
	Bioaugmented	40.75 ± 1.42	0.36 ± 0.02	32.04 ± 0.68	54.50 ± 3.19	0.52 ± 0.04	34.60 ± 0.91

Table 5. Total extents, maximum rates and lag times of ^{14}C-octacosane mineralisation for soil A and B treatments at each timepoint over 341 d. Values represent the mean ± SEM (n = 3).

Aging Period (days)	Soil Treatment	Soil A			Soil B		
		Total extent (%)	Maximum rate (% h^{-1})	Lag time (h)	Total extent (%)	Maximum rate (% h^{-1})	Lag time (h)
0	Control	61.85 ± 1.06	0.40 ± 0.01	28.76 ± 0.80	61.65 ± 0.47	0.55 ± 0.01	42.48 ± 0.87
	Nutrient	66.26 ± 0.81	0.42 ± 0.02	27.09 ± 1.52	37.73 ± 1.25	0.34 ± 0.00	70.43 ± 0.11
	Bioaugmented	65.25 ± 0.53	0.39 ± 0.02	25.54 ± 0.97	32.66 ± 0.42	0.32 ± 0.00	76 95 ± 1.84
31	Control	42.91 ± 0.60	0.27 ± 0.01	20.05 ± 0.73	18.22 ± 0.16	0.15 ± 0.03	37.21 ± 1.21
	Nutrient	43.17 ± 0.44	0.32 ± 0.04	19.35 ± 0.77	27.34 ± 0.37	0.21 ± 0.00	39.29 ± 2.83
	Bioaugmented	47.05 ± 0.80	0.26 ± 0.00	12.93 ± 0.85	21.00 ± 0.55	0.16 ± 0.01	39.38 ± 1.01
62	Control	21.62 ± 0.63	0.14 ± 0.00	40.68 ± 1.20	13.79 ± 0.41	0.08 ± 0.00	75.40 ± 0.78
	Nutrient	34.08 ± 1.04	0.20 ± 0.02	31.76 ± 1.41	18.22 ± 0.13	0.13 ± 0.01	41.78 ± 0.63
	Bioaugmented	48.33 ± 0.37	0.28 ± 0.02	32.25 ± 0.70	20.60 ± 0.42	0.10 ± 0.01	46.86 ± 1.91
124	Control	17.10 ± 0.89	0.13 ± 0.01	48.14 ± 0.75	14.98 ± 0.54	0.13 ± 0.01	64.03 ± 2.02
	Nutrient	37.25 ± 1.00	0.42 ± 0.01	41.52 ± 0.84	12.67 ± 0.75	0.13 ± 0.02	55.88 ± 0.96
	Bioaugmented	48.14 ± 0.97	0.47 ± 0.02	42.65 ± 1.65	18.97 ± 0.68	0.15 ± 0.02	51.78 ± 2.43
341	Control	17.28 ± 0.82	0.09 ± 0.01	59.54 ± 3.30	11.21 ± 0.08	0.07 ± 0.00	75.77 ± 1.18
	Nutrient	41.39 ± 0.52	0.29 ± 0.01	32.82 ± 0.70	12.92 ± 0.31	0.15 ± 0.01	63.45 ± 0.17
	Bioaugmented	43.09 ± 1.98	0.44 ± 0.01	36.50 ± 2.56	14.87 ± 0.35	0.12 ± 0.01	61.66 ± 2.71

Table 6. Ratios of extraction:^{14}C-phenanthrene mineralisation extents using methanol:water and HPCD for soil A and B treatments at each timepoint over 341 d.

Aging period (days)	Soil Treatment	Soil A Ratio extracted: mineralised		Soil B Ratio extracted: mineralised	
		Methanol:water	HPCD	Methanol:water	HPCD
0	Control	0.69	0.71	0.62	0.75
	Nutrient	0.70	0.67	0.52	0.74
	Bioaugmented	0.70	0.75	0.65	0.65
31	Control	0.84	0.74	0.67	0.68
	Nutrient	0.80	0.79	0.82	0.80
	Bioaugmented	0.97	0.97	0.67	0.63
62	Control	0.70	0.49	0.45	0.38
	Nutrient	0.86	0.74	2.41	2.57
	Bioaugmented	0.39	0.90	0.55	0.32
124	Control	0.95	0.63	0.54	0.44
	Nutrient	0.79	0.55	1.47	2.19
	Bioaugmented	0.40	1.06	0.40	0.30
341	Control	0.85	0.53	0.53	0.31
	Nutrient	0.72	0.55	2.35	1.45
	Bioaugmented	0.48	1.25	0.43	0.26

Table1. Physico-chemical properties of soils A and B. Values represent the mean ± SEM (n = 3).

Physico-Chemical Properties	Soil A	Soil B
Moisture content (%)	22.13 ± 1.56	33.12 ±0.22
Bulk density (kg l^{-1})	0.82	0.58
Soil texture	Clay	Sandy clay loam
pH in water	7.47 ± 0.03	6.77 ± 0.09
pH in 0.01 M $CaCl_2$	6.60 ± 0.00	6.10 ± 0.06
Organic carbon (%)	8.50 ± 2.24	15.39 ± 0.84
Organic matter (LOI %)	14.62 ± 3.85	26.47 ± 1.45
DOC (µg ml^{-1})	117.29 ± 14.35	156.01 ± 9.34
TOC (µg g^{-1})	213.43 ± 16.15	251.64 ± 22.99
Extractable nitrogen content (%)	0	0
Extractable phosphorus (%)	0	0
Hydrocarbon Fraction (mg/kg)		
Total Aliphatic	19869	7271
EC ≥ 10 – 12	915	625
EC ≥ 12 – 16	14608	4379
EC ≥ 16 – 35	4256	2259
Total Aromatic	9686.1	11014
EC ≥ 10 – 12	86	58
EC ≥ 12 – 16	1599	1801
EC ≥ 16 – 21	4275	3797
TPH (mg/kg)	29555	18285
Total heterotrophs at timepoint 0 (CFU g^{-1})	$7.1E^7 \pm 1.8E^7$	$9.8E^6 \pm 1.3E^6$
Total degraders at timepoint 0 (CFU g^{-1}):		
- Diesel	$9.9\ E^6 \pm 2.6E^6$	$3.3E^5 \pm 1.0E^5$
- Octacosane	$6.7E^4 \pm 5.8E^4$	$1.1E^5 \pm 4.7E^4$
- Phenanthrene	$4.8E^5 \pm 1.3E^5$	$8.3E^4 \pm 2.9E^4$

Table 7. Ratios of extraction:^{14}C-octacosane mineralisation extents using methanol:water and for soil A and B treatments at each timepoint over 341 d.

Aging period (days)	Soil Treatment	Soil A Ratio extracted: mineralised		Soil B Ratio extracted: mineralised	
		Methanol:water	HPCD	Methanol:water	HPCD
0	Control	0.04	0.06	0.03	0.09
	Nutrient	0.02	0.10	0.05	0.13
	Bioaugmented	0.02	0.07	0.09	0.16
31	Control	0.08	0.23	0.19	0.53
	Nutrient	0.08	0.21	0.20	0.43
	Bioaugmented	0.04	0.11	0.14	0.28
62	Control	0.83	0.59	0.85	0.86
	Nutrient	0.88	0.39	0.45	0.62
	Bioaugmented	0.77	0.31	0.79	0.87
124	Control	1.45	0.80	1.04	1.19
	Nutrient	0.83	0.36	1.29	1.00
	Bioaugmented	0.77	0.32	1.09	1.09
341	Control	1.50	0.84	1.86	1.35
	Nutrient	1.15	0.39	1.38	1.14
	Bioaugmented	0.96	0.41	1.43	1.38