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

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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).

71 ^{14}C-Labelled hydrocarbons may be utilised as a mean to trace their fate and behaviour in
72 complex soil environments under reproducible and controlled conditions (Ortega-Calvo et al.,
73 2013). Furthermore, harsh and exhaustive extraction techniques using solvents, such as
74 dichloromethane (DCM), can be employed to quantify total ^{14}C-hydrocarbon concentrations
75 in soil (Riding et al., 2013). However, DCM extraction measurements have been shown to
76 often overestimate contaminant bioaccessibility in soil, and as such may poorly reflect
77 hydrocarbon biodegradation and bioremediation potential, as well as associated risk
78 (Alexander, 2000; Riding et al., 2013; Adedigba et al., 2018). Conversely, it has been
79 suggested that measurements of the HPCD and methanol:water extracted fractions, as well as
80 the measurement of the mineralisation of ^{14}C-hydrocarbons may provide a more realistic
81 indication of hydrocarbon bioaccessibility and degradability in soils (Patterson et al., 2004;
82 Dew et al., 2005; Adedigba et al., 2018).

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

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

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

106

107 **2. Materials and methods**

108 2.1 Soil and contaminant characteristics

109 Two soils genuinely contaminated with weathered hydrocarbons were collected (top soil; 5-
110 20 cm) from former oil refinery facilities. The soils were labelled as A and B (to maintain
111 owner anonymity), and identified as a clay and sandy loam, respectively. Soil A had
112 previously undergone biopiling treatment, whereas soil B was untreated. The soils were air-
113 dried (24 h) and passed through a 2 mm sieve to remove stones, plant material and facilitate
114 mixing. Prior to spiking, the soils physico-chemical properties were analysed (n = 3)
115 following the methods described by Towell *et al.* (2011a) and are presented in Table 1. In
116 short, soil moisture and loss on ignition (LOI) organic matter contents were determined
117 through oven drying at 105°C and combustion in a furnace at 450°C, respectively, for 24 h.
118 Soil pH was measured in a 1:5 soil (dry wt) to liquid suspension with 0.01 M calcium
119 chloride ($CaCl_2$) solution and then separately using deionised water. Soil organic carbon

120 (OC) content was determined via elemental analysis on a Carlo Erba CHNS- OEA 1108 CN-
121 Elemental Analyser. Acetic acid-extractable phosphorus was measured by shaking soil (0.5 ±
122 0.1 g, dry wt) with 2.5% acetic acid (40 ml), followed by filtration through Whatman 44 filter
123 paper. For extractable ammonium and nitrogen, soil was extracted with 1M KCl. Resultant
124 solutions were then analysed on a flow injection analyser (FIAstar). Total heterotrophic and
125 degrading microbial numbers were enumerated through measurement of colony forming units
126 (CFUs g^{-1} soil, dry wt) on plate count agar and minimal agar supplemented with 0.1% diesel,
127 octacosane or phenanthrene. In brief, soil (1.0 ± 0.1 g, dry wt) was extracted with quarter
128 strength Ringer's solution (10 ml) and, 1 ml of the extract serially diluted with Ringer's
129 solution (0.9 ml). The resultant solutions (0.1 ml) were then spread evenly over agar plates
130 and incubated in the dark at 25 ± 1°C. Colony forming unit counts were performed at 4, 7, 10
131 and 15 d following agar inoculation.

132 Quantification of total petroleum hydrocarbon (TPH) concentrations in each soil was
133 performed using sequential ultrasonic solvent extraction (Risdon et al., 2008). TPH aliphatic
134 and aromatic fractions were assessed through gas chromatography-mass spectrometry (GC-
135 MS) using a Perkin Elmer AutoSystem XL gas chromatograph coupled to a Turbomass Gold
136 mass spectrometer (operated at 70 eV in positive ion mode). The column used was a Restek
137 fused silica capillary column (30 × 0.25 mm internal diameter) coated with RTX®-5MS
138 (0.25 µm film thickness). Splitless injection with a sample volume of 1 µl was applied. The
139 oven temperature was ramped from 60°C to 220°C (at 20°C min^{-1}), then to 310°C (at 6°C
140 min^{-1}) and held at this temperature (for 15 min). For quantitative analysis of target alkanes
141 and PAHs the mass spectrometer was operated using the full scan mode (range *m/z* 50-500).
142 Each hydrocarbon quantification was performed by integrating the peak at specific *m/z*.
143 External multilevel calibrations were carried out for both oil fractions, quantification ranging
144 from 0.5 to 2500 µg ml^{-1} (alkanes) and from 1 to 5 µg ml^{-1} (PAHs). Internal standards for the

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

151

152 2.2 Soil spiking and microcosm preparation

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

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

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

176 Following amendment, the moisture content of all soil treatments (600 g dry wt) was adjusted
177 to 80% WHC using deionised water. Soil treatments were then weighed into amber glass jars
178 (200 g dry wt soil, *n* = 3) and 'aged' in the dark at 21±1°C for the duration of the study. An
179 additional set of microcosms (*n* = 3) consisting of rehydrated soil (200 g dry wt) in amber jars
180 were also prepared as analytical blanks. After 0, 31, 62, 124 and 341 d of incubation, a
181 moisture check was performed on microcosms and soil treatments were sampled and
182 processed as described in the following sections.

183

184 2.3 Determination of total ^{14}C-associated activity in soils

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

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

198

199 2.4 Quantification of extractable ^{14}C-octacosane and ^{14}C-phenanthrene associated activity
200 from soils

201 Extractable fractions of ^{14}C-hydrocarbons were evaluated through three different approaches,
202 where each one can be used as a predictor of the behaviour of the hydrocarbon based on its
203 bioaccessibility (Riding et al., 2013). DCM was used as an exhaustive technique to quantify
204 total extractability/non-extractable hydrocarbon fractions, whilst methanol:water and HPCD
205 represented less aggressive extractions to determine readily extractable and bioaccessible
206 fractions of the ^{14}C-hydrocarbons (Semple et al., 2013; Vázquez-Cuevas and Semple, 2016).
207 At each time point, the extractability of ^{14}C-octacosane or ^{14}C-phenanthrene associated
208 activity in soil treatments was determined by dichloromethane (DCM), methanol:water (1:1
209 v/v) and hydroxypropyl-β-cyclodextrin (HPCD) shake extractions (n = 3). To measure DCM
210 extractability, soil treatments (1.5 g ± 0.1 g wet wt) were grounded with sodium sulphate (8 ±
211 0.1 g) to dry the soil and facilitate DCM-soil interactions. Samples were then transferred into
212 50 ml Teflon centrifuge tubes and 25 ml DCM added to each tube. Methanol:water extraction
213 was performed following the method of Macleod & Semple (2003), where soil treatments (8
214 g ± 0.1 g dry wt) were weighed into Teflon centrifuge tubes (50 ml) and 24 ml methnol:water
215 (1:1 v/v) added to each tube. For HPCD extraction, soil treatments (1.25 g ± 0.1 g wet wt)
216 were mixed with 25 ml 50 mM HPCD solution in 50 ml Teflon centrifuge tubes as per

217 Vázquez-Cuevas & Semple (2016). Tubes were then sealed and all the suspensions shaken
218 for 22 h at 100 rpm on an orbital shaker. Following shaking, soil and supernatants were
219 separated by centrifugation at 3000 x *g* for 1 h. Supernatants for DCM (5 ml), methanol:water
220 (3 ml) and HPCD (6 ml) extractions were sampled, added to Goldstar liquid scintillation
221 cocktail (15 ml) and then counted by LSC as described previously. A mass balance of ≥ 98%
222 was calculated on extraction completion through combustion of extracted soil pellets (1 ± 0.1
223 g) using sample oxidation following the previously described methodology.

224

225 2.5 Mineralisation of ^{14}C-octacosane and ^{14}C-phenanthrene in soils

226 Mineralisation assays based on the soil slurry respirometric approach (Reid et al., 2001;
227 Semple et at., 2006; Vázquez-Cuevas and Semple, 2016) were selected to assess the impact
228 of the treatments towards microbial catabolic response. At each aging period, the
229 mineralisation of remaining ^{14}C-octacosane or ^{14}C-phenanthrene in soil treatments was
230 assessed using respirometric assays as described by Stroud *et al.* (2007b). Respirometric
231 assays (n = 3) were performed in modified 250 ml Schott bottles prepared with 10 g ± 0.2 g
232 soil (wet wt) and 30 ml sterile minimal basal salts medium (MBS). Glass vials (7 ml)
233 containing 1 M NaOH (1 ml) were suspended from the respirometer Teflon-lined lids to trap
234 released $^{14}CO_2$ formed during ^{14}C-hydrocarbon mineralisation. Respirometers containing
235 non-spiked rehydrated soil were also prepared as analytical blanks. The respirometers were
236 then shaken onto an orbital shaker at 100 rpm and 21 ± 1°C, in the dark. Every 24 h over a
237 period of 20 d respirometer $^{14}CO_2$ traps were replaced and Ultima Gold scintillation cocktail
238 (5 ml) added to the sampled vial. Samples were quantified by LSC as previously described.

239

240 2.6 Statistical analysis

241 Following blank correction, statistical analysis of the results was performed using SigmaStat
242 for Windows (Version 2.03 SPSS). Significant effects of treatments (biostimulation and
243 bioaugmentation), hydrocarbon type (phenanthrene and octacosane), and aging time (0, 31,
244 62, 124 and 341 days) on the loss, chemical extractability and mineralisation parameters (lag
245 time, maximum rates and overall extents) of the two ^{14}C-hydrocarbons were evaluated using
246 ANOVAs (Tukey Test) and/or Student t-tests at a 95% confidence level ($p \leq 0.05$). Graphs
247 were presented using SigmaPlot 2000 for Windows (Version 10.0, SPSS Inc.).

248

249 **3. Results**

250 3.1 Loss of ^{14}C-phenanthrene and ^{14}C-octacosane residues from soil

251 Loss of ^{14}C-activity from the soils was assessed at each time point for both of the
252 hydrocarbons under control and treatment regimens (Tables 2 and 3; SM-1). The greatest
253 losses of ^{14}C-phenanthrene was observed in the control and biostimulated treatments for soil
254 A and B, respectively. Total ^{14}C-phenanthene associated activity remaining in control soils
255 decreased by 79 % (soil A) and 55% (soil B) between 0 and 341 d. The initial rapid rate of
256 loss of ^{14}C-residues was 1.03 % d^{-1} within the first 62 days, followed by a slower rate of loss
257 of 0.01 % d^{-1} (62 – 341 d). Although losses of ^{14}C-activity were observed to be significantly
258 higher in the control soil A than B, no significant differences ($p \geq 0.05$) in the rate and loss of
259 ^{14}C-phenanthrene residues were apparent (Table 2). Furthermore, bioaugmentation had a
260 negative impact upon the loss of ^{14}C-phenanthrene residues from both soils, with total ^{14}C-
261 phenanthrene activity remaining predominantly, but not exclusively, significantly higher ($p \leq$
262 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).

285 3.2 Extractability of ^{14}C-phenanthrene and ^{14}C-octacosane associated activity from soils

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

305 For the biostimulation and bioaugmentation treatments, overall, no significant trends ($p \geq$
306 0.05) in ^{14}C-phenanthrene extraction (by methanol:water and HPCD) were determined
307 between control and treated soil B, and control and nutrient treated soil A (Table 2).
308 However, for bioaugmented soil A, ^{14}C-phenanthrene extraction by methanol:water was
309 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

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

337 Overall, results showed that with increasing soil incubation time, the DCM-extractability of
338 ^{14}C-phenanthrene and ^{14}C-octacosane significantly decreased ($p \leq 0.05$), indicating that aging
339 was taking place (SM-1 and SM-2). In addition, and as expected, significantly lower ($p \leq$
340 0.05) amounts of ^{14}C-phenanthrene and ^{14}C-octacosane were extracted from soils by
341 methanol:water and HPCD (Table 2 and 3).

342

343 3.3 ^{14}C-Phenanthrene and ^{14}C-octacosane biodegradation in soils

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

353 When analysing the behaviour of both soils across time, at 0 d significantly less ($p \leq 0.05$)
354 ^{14}C-phenanthrene was mineralised in soil B, compared to soil A (Table 4; SM-2). At this
355 stage (0 d ageing), lag times were also significantly longer ($p \leq 0.05$) and maximum rates of
356 ^{14}C-phenanthrene mineralisation lower for soil B. For instance, while soil A had a lag phase
357 of 28.76 h in soil B took 42.48 h to mineralise >5% of the ^{14}C-activity in the control soil.
358 This trend was not observed over the whole aging period, with maximum rates and extents of

359 ^{14}C-phenanthrene mineralisation between control soils often being statistically similar ($p \geq$
360 0.05) at subsequent ageing times (Table 4; SM-2). In this study, soil A contained
361 considerably higher TPH (1.6 times more) and phenanthrene concentrations (798 mg kg^{-1}),
362 and had a larger phenanthrene degrading population than soil B (Table 1). These observations
363 are also supported by^{14}C-phenanthrene lag times remaining significantly longer ($p \leq 0.05$) for
364 soil B (than soil A).

365 For the aliphatic hydrocarbon, comparisons between ^{14}C-mineralisation parameters (lag
366 times, maximum rates and extents of mineralisation) indicated that ^{14}C-octacosane was less
367 readily degraded by the microflora of both soils (Table 5; SM-3). In general, maximum rates
368 and extents of ^{14}C-octacosane mineralisation were significantly lower ($p \leq 0.05$) compared to
369 ^{14}C-phenanthrene, even though the soil microflora had been previously exposed to high
370 aliphatic hydrocarbon concentrations (≥7271 mg kg^{-1}) (Table 5; SM-3). Similar findings were
371 observed for biostimulated and bioaugmented soils (Table 5; SM-3). Lag times for
372 comparable soil treatments were also often significantly higher ($p \leq 0.05$) for ^{14}C-octacosane,
373 compared to ^{14}C-phenanthrene, further suggesting reduced catabolic behaviour toward this
374 hydrocarbon.

375

376 3.3.1 Impact of biostimulation and bioaugmentation

377 In this study, biostimulation and bioaugmentation treatments generally had no impact upon
378 the degradation of ^{14}C-phenanthrene in soils over 341 d (Table 4; SM-2). ^{14}C-Phenanthrene
379 mineralisation parameters were predominately (but not exclusively) statistically similar ($p \geq$
380 0.05) between control, nutrient treated and bioaugmented soils, at aging times (Table 4). The
381 exception to this was nutrient treated soil B without ageing (0 d), where lag times were higher
382 and extents/maximum rates of ^{14}C-phenanthrene mineralisation significantly lower ($p \leq 0.05$),

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

387 Conversely, biostimulation and bioaugmentation significantly increased ($p \leq 0.05$) ^{14}C-
388 octacosane mineralisation in soil A, but generally only after 31 d ageing (Table 5; SM-3).
389 High ^{14}C-octacosane associated catabolic activity was further reflected by lag times being
390 about 8 h shorter for treated soils, than the control, after 31 d ageing. Between treatments,
391 ^{14}C-octacosane mineralisation was often (but not exclusively) significantly higher ($p \leq 0.05$)
392 for bioaugmented soil A, as opposed to nutrient treated (Table 5; SM-3). These findings also
393 correspond with the significantly lower ($p \leq 0.05$) amounts of ^{14}C-octacosane associated
394 activity remaining in bioaugmented soil A (compared to control and nutrient treated), after
395 341 d. It is suggested that although comparably rapid and extensive mineralisation (≥ 60%)
396 and ^{14}C-octacosane residue loss was attained by the indigenous microflora of soil A, between
397 0-31 d, the presence of specialised degraders was required to sustain degradation with
398 increasing aging time, and facilitate octacosane removal from this soil.

399 Similarly, in soil B bioaugmentation significantly increased ($p \leq 0.05$) the rates and extents of
400 ^{14}C-octacosane mineralisation and reduced lag times in respect to controls, at 62, 124 and 341
401 d (SM-3). Bioaugmentation had considerably less impact on ^{14}C-octacosane degradation in
402 soil B, where extents of ^{14}C-octacosane mineralised were significantly lower ($p \leq 0.05$) for
403 bioaugmented soil B, than A, at aging times (Table 5; SM-3). As per previously discussed
404 these findings may be attributed to the putative impact of OM/OC on the bioaccessibility of
405 ^{14}C-octacosane for degradation purposes in this soil.

406

407

408 3.4 Comparisons between methanol:water and HPCD-extractability and mineralisation of
409 ^{14}C-phenanthrene and ^{14}C-octacosane

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

414 In general, both methanol:water and HPCD extraction underestimated the mineralisation of
415 ^{14}C-phenanthrene in soils over 341 d. At aging times, total amounts of ^{14}C-phenanthrene
416 mineralised were often significantly higher ($p \leq 0.05$) than amounts extracted (by both
417 methanol:water and HPCD) for treated and control soils. This was further reflected by
418 methanol:water and HPCD extraction:mineralisation ratios for treated and control A and B
419 soils being predominantly higher than 0.80, over the aging period (Table 6). Although, for
420 nutrient treated soil B, both extraction methods were determined to significantly overestimate
421 ($p \leq 0.05$) ^{14}C-phenanthrene mineralisation at 62, 124 and 341 d. The exception to these
422 findings was HPCD extraction for bioaugmented soil A, which was determined to strongly
423 correlate with the ^{14}C-phenanthrene mineralisation values in this soil at 31, 62 and 124 d.
424 Furthermore, over 341 d the average HPCD extraction:mineralisation ratio for bioaugmented
425 soil A was 0.99, as opposed to 0.62 and 0.66 for control and nutrient treated soil A,
426 respectively; and 0.51, 1.55 and 0.41 for control, nutrient treated and bioaugmented soil B.

427 Similarly, for ^{14}C-octacosane there was much variability in both methanol:water and HPCD
428 extraction:mineralisation ratios for control and treated soils over the aging period, with poor
429 correlation to mineralisation generally observed (Table 7). At 0, 31 and 62 d, both extraction
430 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

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

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

470

471 4.2 ^{14}C-Phenanthrene and ^{14}C-octacosane degradation in soils

472 In this study, the indigenous microflora of both soils demonstrated high catabolic potential to
473 degrade ^{14}C-phenanthene. The biodegradation of petroleum hydrocarbons is widely
474 acknowledged to vary between different hydrocarbons, with physico-chemical properties
475 such as molecular structure, size, hydrophobicity and solubility suggested to strongly
476 influence their degradation potential (Riding et al., 2013; Yu et al., 2018). Moreover, the
477 general increase of mineralisation of ^{14}C-phenanthene suggests that a s period of microbial
478 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.

551 4.3 Extractability of ^{14}C-phenanthrene and ^{14}C-octacosane associated activity from soils

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

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

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

576 Riding et al., 2013). Furthermore, correlations between decreasing contaminant
577 bioaccessibility due to increased sorption in soils with higher SOM concentration and aging
578 have been reported (Jones and Edwards, 1998; Nam et al., 1998; Yang et al., 2010; Luo et al.,
579 2012).

580 Methanol:water and HPCD extractability was lower when evaluating ^{14}C-octacosene than in
581 the case of ^{14}C-phenanthrene. Hydrophobicity is thought to significantly influence
582 contaminant mobility and biological availability in soil, with hydrophobic contaminants
583 typically observed to be more strongly sorbed to the soil matrix (Löser et al., 1999; Reid et
584 al., 2000; Gunasekara and Xing, 2003). Due to octacosane having a more hydrophobic nature,
585 it is plausible it may partition more strongly within the solid soil phases and thus be less
586 readily extracted, compared to phenanthrene. Interestingly, as opposed to ^{14}C-phenanthrene,
587 ^{14}C-octacosane extraction (by both methanol:water and HPCD) increased over the ageing
588 period. This indicates an increase in bioaccessibility and possible contaminant remobilisation
589 over the aging period (Riding et al., 2013). Due to the low amounts of ^{14}C-octacosane
590 extracted at 0 and 31 d (for all soils and treatments), another plausible explanation is low
591 methanol:water and HPCD extraction efficiency for this ^{14}C-hydrocarbon (Reid et al., 2000),
592 as it will be further discussed.

593

594 4.3.1 Comparisons between methanol:water and HPCD-extractability and mineralisation of
595 ^{14}C-phenanthrene and ^{14}C-octacosane in soils

596 There has been considerable research surrounding the development of less exhaustive
597 extraction techniques to estimate the bioaccessibility and accurately predict the degradability
598 of organic contaminants in soil (Reid et al., 2000; Stokes et al., 2005; Rhodes et al., 2008).
599 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,

624 soil-contaminant contact time and the presence and activity of degrading microorganisms. In
625 this study two contrasting hydrocarbons were analysed. As such, the PAH (phenanthrene) and
626 the long-chained aliphatic hydrocarbon (octacosane) exhibited contrasting behaviours. Due to
627 its extremely hydrophobic nature, octacosane showed to be more influenced by SOM/OC,
628 having a higher inhibitory impact upon the bioavailability/bioaccessibility and its microbial
629 degradation in soils. Results from this study clearly show that microbial degradation should
630 not be considered as a cost-effective remediation approach without a more comprehensive
631 evaluation of the partitioning, extractability and bioaccessibility of hydrocarbons in soil.
632 Contrary to what is typically done, determining the likelihood of effective biodegradation by
633 the stimulation of indigenous microorganisms or through bioaugmentation needs to be
634 assessed by both chemical and biological measurements of bioaccessibility, rather than just
635 by which is totally extractable from soil. However, specific characteristics of the soil must be
636 considered to select the proper methods as in the case of soils with high loadings of organic
637 matter and/or organic contaminants which might prevent accurate assessment of contaminant
638 bioaccessibility, as measured by HPCD. Considering this, it is proposed that a combined and
639 integrated biological/chemical approach is required to fully assess the bioremediation
640 potential and thus determine suitable clean up targets for petroleum-contaminated sites.
641

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651 References

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

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

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

662 Anyanwu, I.N., Semple, K.T., 2018. Impact of single and binary mixtures of phenanthrene
663 and N-PAHs on microbial utilization of 14C-glucose in soil. Soil Biol. Biochem. 120,
664 222–229. https://doi.org/10.1016/j.soilbio.2018.02.009

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

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

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

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

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

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

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

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

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

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

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

699 introduction of a phenanthrene-LNAPL mixture into field-wet soil. Environ. Pollut. 126,
700 399–406. https://doi.org/10.1016/S0269-7491(03)00230-6

701 Duan, L., Naidu, R., Liu, Y., Palanisami, T., Dong, Z., Mallavarapu, M., Semple, K.T.,
702 2015a. Effect of ageing on benzo[a]pyrene extractability in contrasting soils. J. Hazard.
703 Mater. 296, 175–184. https://doi.org/10.1016/j.jhazmat.2015.04.050

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

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

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

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

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

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

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

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

725 Jiang, Y., Brassington, K.J., Prpich, G., Paton, G.I., Semple, K.T., Pollard, S.J.T., Coulon, F.,
726 2016. Insights into the biodegradation of weathered hydrocarbons in contaminated soils
727 by bioaugmentation and nutrient stimulation. Chemosphere 161, 300–307.
728 https://doi.org/10.1016/j.chemosphere.2016.07.032

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

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

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

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

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

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

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

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

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

752 Macleod, C.J.A., Semple, K.T., 2002. The adaptation of two similar soils to pyrene
753 catabolism. Environ. Pollut. 119, 357–364. https://doi.org/10.1016/S0269-
754 7491(01)00343-8

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

758 Masy, T., Demanèche, S., Tromme, O., Thonart, P., Jacques, P., Hiligsmann, S., Vogel, T.M.,
759 2016. Hydrocarbon biostimulation and bioaugmentation in organic carbon and clay-rich
760 soils. Soil Biol. Biochem. 99, 66–74. https://doi.org/10.1016/j.soilbio.2016.04.016

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

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

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

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

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

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

783 Patterson, C.J., Semple, K.T., Paton, G.I., 2004. Non-exhaustive extraction techniques
784 (NEETs) for the prediction of naphthalene mineralisation in soil. FEMS Microbiol. Lett.
785 241, 215–220. https://doi.org/10.1016/j.femsle.2004.10.023

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

831 Semple, K.T., Dew, N.M., Doick, K.J., Rhodes, A.H., 2006. Can microbial mineralization be
832 used to estimate microbial availability of organic contaminants in soil? Environ. Pollut.
833 140, 164–172. https://doi.org/10.1016/j.envpol.2005.06.009

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

837 Semple, K.T., Doick, K.J., Wick, L.Y., Harms, H., 2007. Microbial interactions with organic
838 contaminants in soil: Definitions, processes and measurement. Environ. Pollut. 150,
839 166–176. https://doi.org/10.1016/j.envpol.2007.07.023

840 Semple, K.T., Morriss, a. W.J., Paton, G.I., 2003. Bioavailability of hydrophobic organic
841 contaminants in soils: fundamental concepts and techniques for analysis. Eur. J. Soil Sci.
842 54, 809–818. https://doi.org/10.1046/j.1365-2389.2003.00564.x

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

846 Škulcová, L., Neuwirthová, N., Hofman, J., Bielská, L., 2016. Assessment of the biological
847 and chemical availability of the freshly spiked and aged DDE in soil. Environ. Pollut.
848 212, 105–112. https://doi.org/10.1016/j.envpol.2016.01.042

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

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

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

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

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

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

867 1253. https://doi.org/10.1111/j.1365-2672.2007.03401.x

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

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

874 Towell, M.G., Paton, G.I., Semple, K.T., 2011b. The biodegradation of cable oil components:
875 Impact of oil concentration, nutrient addition and bioaugmentation. Environ. Pollut. 159,
876 3777–3783. https://doi.org/10.1016/j.envpol.2011.06.043

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

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

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

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

888 Vázquez-Cuevas, G.M., Semple, K.T., 2016. Measurement of Hydrocarbon Bioavailability in
889 Soil, in: McGenity, T., Timmis, K., Nogales, B. (Eds.), Hydrocarbon and Lipid
890 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.

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

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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 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

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

TEMPORAL CHANGES IN THE BIOAVAILABILITY AND BIODEGRADATION OF TARGET HYDROCARBONS IN SOILS FROM FORMER REFINERY FACILITIES

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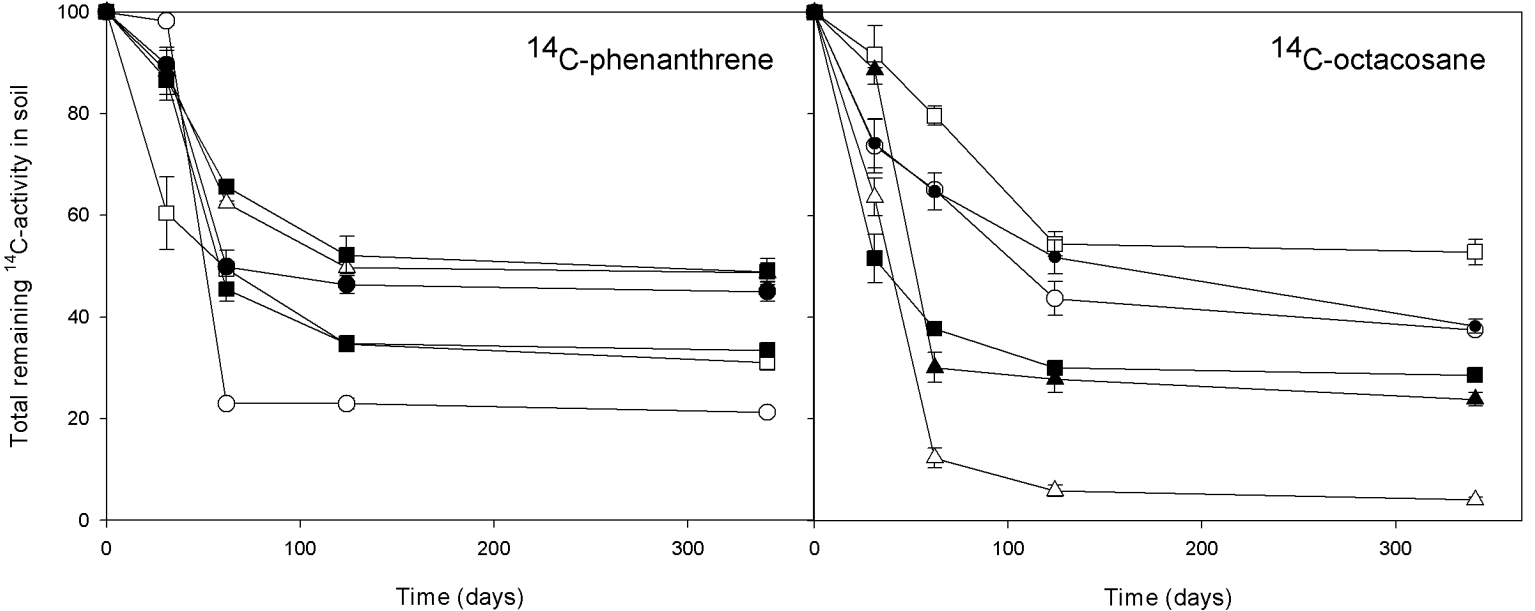
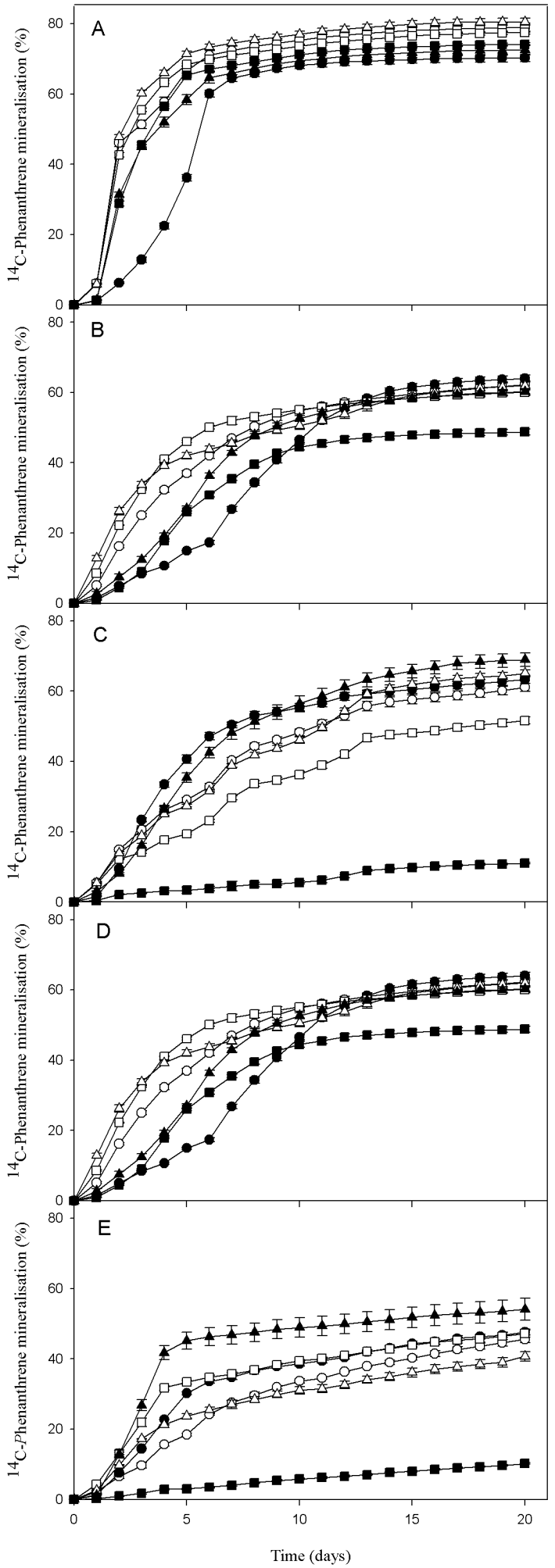
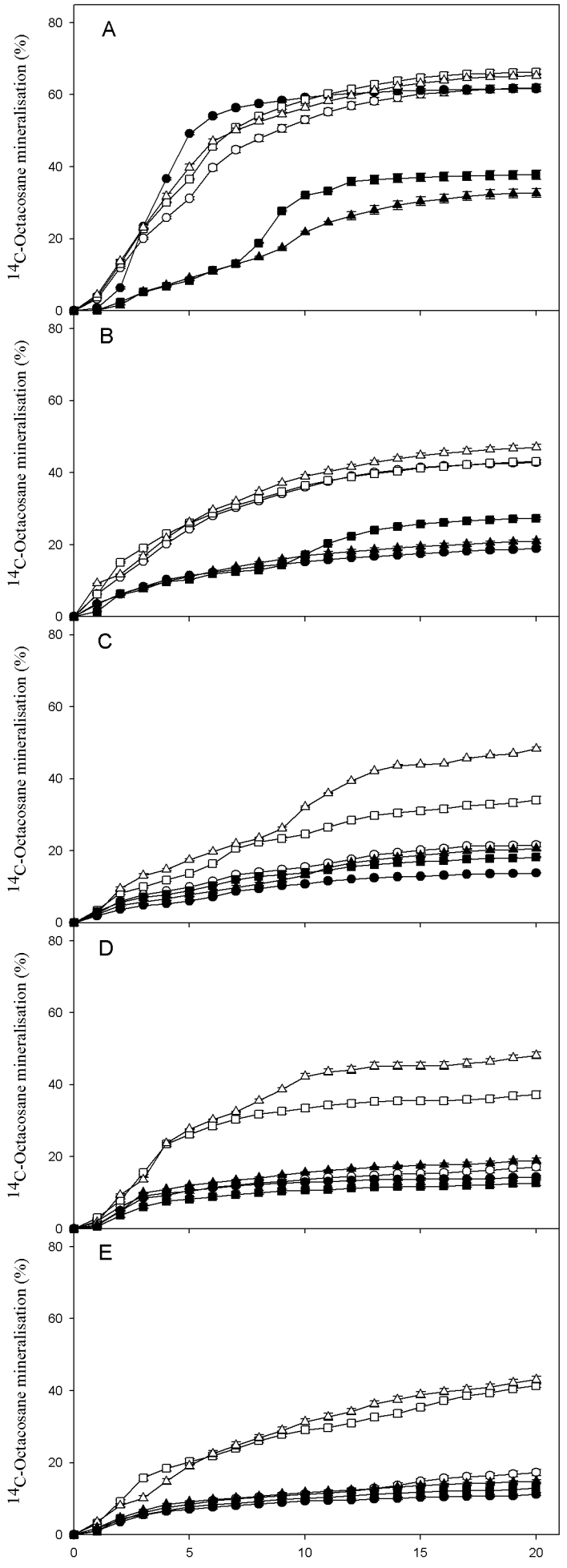
Figure 1. Loss of ^{14}C-phenanthrene and ^{14}C-octacosane residues from soil A, control (○), biostimulation (nutrient amended) (□), bioaugmentation (microbial inoculum amended) (Δ); and soil B, control (●), biostimulation (nutrient amended) (■), bioaugmentation (microbial inoculum amended) (▲) over 341 d. Values represent the mean ± SEM (n = 3).

Figure 2. Mineralisation of ^{14}C-phenanthrene for soil A, control (○), nutrient (□) and bioaugmentation (Δ); and soil B control (●), nutrient (■) and bioaugmentation treatments (▲); at (A) 0, (B) 31, (C) 62, (D) 124 and (E) 341 d. Error bars are 1 SEM (n = 3).

Figure 3. Mineralisation of 14C-octacosane for soil A, control (○), nutrient (□) and bioaugmentation (Δ); and soil B control (●), nutrient (■) and bioaugmentation treatments (▲); at (A) 0, (B) 31, (C) 62, (D) 124 and (E) 341 d. Error bars are 1 SEM (n = 3).


Total remaining 14C-activity in soil
100
80
60
40
20
0
0
100
200
300
Time (days)
14C-phenanthrene
0
100
200
300
Time (days)
14C-octacosane


A
B
C
D
E
^{14}C-Phenanthrene mineralisation (%)
0
20
40
60
80
0
5
10
15
20
Time (days)


A
B
C
D
E
^{14}C-Octacosane mineralisation (%)
0
20
40
60
80
0
5
10
15
20
Time (days)