Lancaster Environment Centre

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BIODEGRADATION OF ALIPHATIC AND POLYCYCLIC AROMATIC HYDROCARBONS IN PETROLEUM OIL-CONTAMINATED SOILS

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Declaration

I hereby declare that the body of work presented in this thesis is my own original work and no part of it has been submitted in substantially the same form for the award of a higher degree elsewhere.

Olusoji Olusegun Igunnugbemi

Abstract

Crude petroleum and its refined products are principal energy sources driving industrialisation; albeit, the resultant pollution of the natural environment is antithetical to sustainable development. Soil pollution arising from the release of petroleum hydrocarbons causes changes in the soil microbial community structure and functional diversity, which can result in significant impact on soil ecosystem functioning. A number of physical, chemical and biological processes determine hydrocarbon fate in soil, but microbial degradation is generally considered to be the most important loss process. The overall aim of this project was to assess the biodegradation of aliphatic and polycyclic aromatic hydrocarbons in soils contaminated with petroleum oil. The effects of the presence and concentration of co-contaminants, the presence and concentration of HP-β-CD, and of prescribed fire on microbial catabolic activity in soils were investigated. Selected soils from the UK and the Antarctic (SOM <1–27% and clay <0.01–42%) provided a good basis for evaluating the influence of soil physicochemical properties. Levels of hydrocarbons in the background soils reflect their proximity to anthropogenic input sources. Indigenous mineralisation of target hydrocarbons was measured using ^{14}C-radiorespirometry in the background soils and soils amended with increasing diesel oil concentrations analogous to the spatial heterogeneity in contaminant levels common at polluted sites. The ^{14}C-hydrocarbons used were naphthalene, phenanthrene, benzo[a]pyrene, hexadecane and octacosane. Collectively, the results from studies of effect of diesel concentration highlight the need to consider the potential variations in the development of indigenous catabolic activity towards various hydrocarbons when designing bespoke remediation strategies, as contaminant levels varied widely, even on a millimetre/centimetre-scale, in polluted soils. The efficiency of microbial utilisation of labile C-substrate was also influenced by the initial diesel concentration and soil contact time; the use of *in-situ* derived k_{EC} values is advised to quantify microbial biomass-C in petroleum oil-polluted soils. The failure of HP-β-CD to promote greater benzo[a]pyrene mineralisation despite a significantly enhanced solubilisation has weighty ecotoxicological implications for cyclodextrin-aided bioremediation of PAH-impacted soil. The negligible long-term effects on overall microbial activity and PAH degradative ability of soils and the substantial reduction of contaminant burdens in soil suggest low-severity prescribed fire as a practicable first-line option for remediation of heavily oil-polluted soils.

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SOJ, indeed this is just the beginning of the journey, stay focus and never depart from your Source.

To the 'One who does what He says' – my Source, my Sustenance, my Strength and my Salvation, I forever pledge my life oh Lord. The deal is intact, thank You.

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Abbreviations

AHC	Aliphatic hydrocarbon
BATNEEC	Best alternative technology not entailing excessive cost
BPEO	Best practicable environmental option
BTEX	Benzene, toluene, ethylbenzene, xylene
C_{effect}	Concentration effect
CFU	Colony forming unit
GC-FID	Gas chromatography-flame ionisation detector
GC-MS	Gas chromatography-mass spectrometry
HMW	High molecular weight
HOC	Hydrophobic organic compound
K_{ow}	Octanol-water partition coefficient
LMW	Low molecular weight
MBS	Minimal basal salt
mb d^{-1}	Million barrel per day
NAPL	Non aqueous phase liquid
OPEC	Organisation of the Petroleum Exporting Countries
PAH	Polycyclic aromatic hydrocarbon
POP	Persistent organic pollutant
ppm	Parts per millions
rpm	Revolutions per minute
TPH	Total petroleum hydrocarbon
US EPA	United States Environmental Protection Agency

Thesis format – List of papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. **Igunnugbemi, O.O.,** Semple, K.T. Enhancing benzo[a]pyrene bioaccessibility and biodegradation: applicability and limitations of solubility enhancement agents in bioremediation of soil and sediment. *For submission to Journal of Hazardous Materials.*

II. **Igunnugbemi, O.O.,** Swallow, N., Semple, K.T. Impact of diesel oil concentrations and soil contact time on naphthalene and benzo[a]pyrene mineralisation in soil. *Submitted to Journal of Hazardous Materials.*

III. **Igunnugbemi, O.O.,** Ikediashi, M.N., Swallow, N., Semple, K.T. The effects of diesel concentration and soil contact time on the development of indigenous catabolic activities toward hydrocarbons. *For submission to International Biodeterioration & Biodegradation.*

IV. **Igunnugbemi, O.O.,** Ikediashi, M.N., Semple, K.T. Effect of diesel oil concentration on microbial utilisation of ^{14}C-glucose in soil. *For submission to FEMS Microbiology Letters.*

V. **Igunnugbemi, O.O.,** Okere, U.V., Semple, K. T. Biodegradation of phenanthrene and benzo[a]pyrene under complex contaminant systems in soils. *Submitted to Environmental Pollution.*

VI. **Igunnugbemi, O.O.,** Abbruzzese V., Semple, K.T. Effects of HP-β-CD concentrations and repeated exposures to diesel on biodegradation of benzo[a]pyrene in soil. *For submission to Environment International.*

VII. **Igunnugbemi, O.O.,** Abbruzzese V., Semple, K.T. Influence of HP-β-CD-enhanced solubilisation and diesel-enhanced catabolic activity on

benzo[a]pyrene biodegradation in four soils. *For submission to Environmental Science & Technology.*

VIII. **Igunnugbemi, O.O.,** Semple, K.T. There is no relationship between endpoint mineralisation rate and amount of benzo[a]pyrene residues that remained bioaccessible in soil slurries. *For submission to Biodegradation.*

IX. **Igunnugbemi, O.O.,** Semple, K.T. Biodegradability of naphthalene, phenanthrene and benzo[a]pyrene in diesel oil-contaminated soil after exposure to prescribed fire. *For submission to Chemosphere.*

X. **Igunnugbemi, O.O.,** Semple, K.T. Effect of diesel oil concentration on the quantification of ^{14}C-biomass in different soils. *For submission to Soil Biology and Biochemistry.*

XI. **Igunnugbemi, O.O.,** Semple, K.T. Short-term microbial turnover of labile carbon in diesel oil-contaminated soils: influences of diesel concentration and soil texture. *For submission to Soil Biology and Biochemistry.*

Appendix

XII. Ite, A.E., **Igunnugbemi, O.O.,** Hanney, N., Semple, K.T. The effect of rhizosphere soil and root tissue amendment on microbial mineralisation of target ^{14}C-hydrocarbons in contaminated soil. *For submission to Chemosphere.*

Introduction to thesis

1.1. Petroleum oil pollution of soil environments

Crude petroleum and its refined products provide a substantial energy source to drive the heavy engines of industrialisation; albeit, the resultant pollution of the natural environment is antithetical to sustainable development. Humanity is continuously been enmeshed in clouds of emissions and streams of effluents from both mobile machines that crisscross, and immobile installations that dot the landscape of our cities and rural areas. Leaks and accidental spills may occur during crude petroleum exploration onshore or offshore, but soil pollution during refining seems to be of minor importance since most of the process is carried out in closed systems (Cerniglia, 1992). Principally, the large-scale anthropogenic pollution of soil occurs during storage and transportation as a result of petroleum oil spills, and at railroad yards or filling stations during refuelling of vehicles (Wilson and Jones, 1993). Careless handling during disposal of oil wastes may also contribute to the burden of petroleum oils in the soil environment. It is difficult to quantify the actual pollution of the terrestrial environment by oil because some of the incidents are unintentional (e.g., accidental spillage from oil tankers); it is estimated that over one million tonnes of oil are spilled into UK terrestrial ecosystems every year (Ripley *et al.*, 2002). Natural crude petroleum seepage is estimated to be 600,000 metric tonnes per year with a range of uncertainty of 200,000 metric tonnes per year (Das and Chandran, 2011).

Crude petroleum and its derived oils, such as diesel oil, contain complex mixtures of hydrophobic organic compounds (HOCs) – asphaltenes, aliphatic hydrocarbons (AHCs) and polycyclic aromatic hydrocarbons (PAHs) which are potentially recalcitrant and accumulate in the environment (Wang and Bartha, 1990). High concentrations of petroleum oils in soil represent both ecological and ecotoxicological

risks as oils contain both aliphatic and aromatic hydrocarbons, some of which possess toxic, carcinogenic and mutagenic properties, and can persist in soil. Specifically, PAHs are of the largest threat to human health due to their added resistance to biological, chemical and photolytic breakdown (Semple *et al.*, 2003); as of January 2008, the United States Environmental Protection Agency (US EPA) designated 28 PAHs as priority pollutants (Gan *et al.*, 2009). Accidental leakages or large-scale spills of petroleum oils can significantly impact on vast expanses of sensitive ecosystems with enormous effects on wildlife and the human society; severe damages to aquatic and terrestrial habitats, injuries and sometimes death of plants and animals have been reported (George *et al.*, 2011). A recent review highlights the effects of exposure to spilled oils on human health to range from acute physical effects, such as vegetative-nervous symptoms, skin and mucous irritations to psychological trauma and, to genotoxic and endocrine effects in exposed individuals (Aguilera *et al.*, 2010).

1.2. Physicochemical and biological properties of petroleum hydrocarbons

As the main components of fuels and oils, AHCs are a group of non-aromatic and non-cyclic hydrocarbons. Structurally, they can be sub-divided as: (i) alkanes – saturated hydrocarbons with C–C bonds; (ii) alkenes – unsaturated hydrocarbons containing double C=C bonding; and (iii) alkynes – unsaturated hydrocarbons containing a triple C≡C bond (Stroud *et al.*, 2007b). Table 1 shows the physicochemical properties of selected AHCs. Mid-length (C_{14}–C_{20}) alkanes are non-polar, virtually water insoluble with increasing melting and boiling points as carbon number increases within the molecule. Most alkanes exist as either non-aqueous phase liquids (NAPLs) (e.g. hexadecane) or solids (e.g. octacosane) at room temperature, making them not readily volatilised or leached from soil (Aislabie *et al.*, 2008; Serrano *et al.*, 2008). Compared

51 to PAHs of similar molecular weight or hydrophobicity (e.g., chrysene or
52 dibenz[*a,h*]anthracene *vs.* hexadecane), AHCs are likely to be more susceptible to
53 microbial degradation in soils (Stroud *et al.*, 2007a; Wentzel *et al.*, 2007). This is
54 largely due to their related analogous structures to many lipids and/or fatty acids
55 occurring naturally in the environment; hence, the enzymatic pathways for AHCs
56 degradation are often constitutive in the soil microorganisms. However, the extent of
57 biodegradation of AHCs observed in soils is typically lower than their PAH
58 counterparts (Chaineau *et al.*, 1995); this being the greater effect of the factors of
59 bioaccessibility on AHCs than PAHs.
60 PAHs consist of two or more fused benzene rings and/or pentacyclic molecules that are
61 arranged in linear, angulate or clustered arrays (Cerniglia, 1992). Although
62 anthropogenic sources significantly contribute to PAHs found as environmental
63 contaminants, natural processes, such as bush fires, and during thermal geologic
64 events, add to the PAH burdens in soils (Bamforth and Singleton, 2005). PAHs have
65 been detected in various environmental media including air (Kim *et al.*, 2013), water
66 and sediment (Lewis *et al.*, 2011), and soil (Wilcke, 2007). The ubiquitous distribution
67 of PAHs in soils is partly because they are also products of incomplete combustion
68 (Kim *et al.*, 2013), or produced biogenically by microorganisms associated with
69 termites (Musa Bandowe *et al.*, 2009) and partly due to their persistence and increased
70 chemical recalcitrance in soil (Bamforth and Singleton, 2005). The amounts of PAHs
71 can range from a few $\mu g\ kg^{-1}$ to hundreds of $g\ kg^{-1}$ in soils, depending on the source of
72 pollution (Kanaly and Harayama, 2000). The physicochemical properties of PAHs, to a
73 large extent, govern their bioavailability/bioaccessibility, biodegradability or
74 recalcitrance and acute toxicity or mutagenicity/genotoxicity. PAH bioaccessibility
75 (which is determined by aqueous solubility and hydrophobicity) as well as

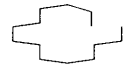
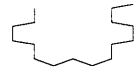
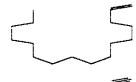
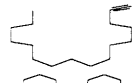
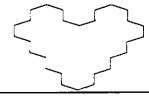
biodegradability decreases as aromatic ring number increases; also volatility decreases with increasing ring number. Apparently, acute toxicity decreases and mutagenicity increases as ring number increases; higher-molecular-weight (HMW)-PAHs exhibit lower toxicity than lower-molecular-weight (LMW)-PAHs mainly because of decreased solubility (Sverdrup *et al.*, 2002). Most HMW-PAHs are believed to possess carcinogenic and mutagenic properties (Hu *et al.*, 2012). Some of the physicochemical properties of the 16 US EPA priority PAHs are presented in Table 2. Excellent reviews on the physicochemical properties and genotoxicity, as well as biodegradability of various AHCs and PAHs are available in the literature (e.g., Juhasz and Naidu, 2000; Stroud *et al.*, 2007b; Seo *et al.*, 2009; Kim *et al.*, 2013). Here, a brief discussion of the selected AHCs and PAHs used in the studies presented in this thesis is undertaken.

1.2.1 Hexadecane and Octacosane

Hexadecane ($n C_{16}H_{34}$) and octacosane ($n C_{28}H_{58}$), which are saturated AHCs with C–C bonds, have molecular weights of 226.44 and 394.77 g mol^{-1}, respectively (Table 1). In general, AHCs have high *n*-octanol–water partition coefficient (log K_{ow}) values (e.g., that of hexadecane is 9.1), indicating that the compounds are highly hydrophobic and virtually insoluble in water. Along with other AHCs and PAHs, hexadecane and octacosane are usually present in petroleum oils. Hexadecane exists as a liquid while octacosane is a white powdery or waxy solid at room temperature; although not individually produced industrially, hexadecane is found in several products and can be used as lamp oil and as a solvent. Although almost insoluble in the aqueous phase, biodegradation of hexadecane or octacosane can be relatively rapid and extensive in water and soil matrices (Aislabie *et al.*, 2008; Bouchez-Naitali and Vandecasteele, 2008; Towell *et al.*, 2011a). The rapid mineralisation of hexadecane to CO_2 is thought

to be due, in part, to the constitutive nature of the enzymatic pathways used for its biodegradation, and in part, to the ability of microorganisms to adapt via certain specialised mechanisms to enhance the passive uptake of poorly-soluble AHCs (Whyte *et al.*, 1998; Stroud *et al.*, 2007b). These mechanisms include the hydrophobic nature of microbial cell walls to facilitate direct cell contact with the AHCs and/or the production of biosurfactants to promote higher solubilisation of the AHCs (Bouchez-Naïtali *et al.*, 2001; Stroud *et al.*, 2007b; Bouchez-Naitali and Vandecasteele, 2008).

Table 1: Physicochemical properties of selected aliphatic hydrocarbons (Howard and Meyln, 1997; Stroud *et al.*, 2007b).

Aliphatic	MW[a]	Structure	mp[b]	bp[c]	Sol[d]	K_{ow}[e]
Tetradecane	198.38		5.5	253	0.0003	7.2
Hexadecane	226.64		18	287	0.0009	9.1
Hexadecene	224.43		3–5	274	N/A	N/A
Hexadecyne	222.42		15	148	N/A	N/A
Octacosane	394.77		57–62	278	N/A	N/A

[a] MW: molecular weight (g mol^{-1}) [b] mp: melting point (°C); [c] bp: boiling point (°C); [d] Sol: aqueous solubility (mg l^{-1}); [e] log K_{ow}: logarithm of the *n*-octanol–water partitioning coefficient.

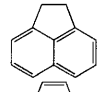
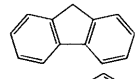
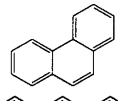
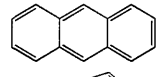
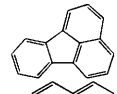
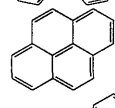
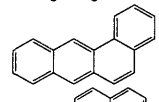
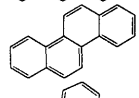
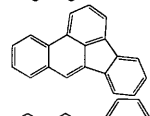
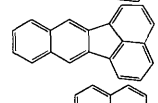
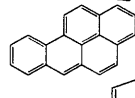
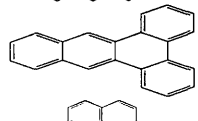
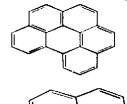
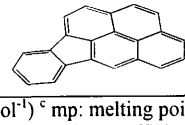
A large number of bacteria and fungi, as well as some algae capable of using AHCs as carbon and energy source have been described. Wentzel *et al.* (2007) provides a detailed listing of isolated bacterial strains characterised for the degradation of long-chain *n*-alkanes. Bacteria able to rapidly degrade and mineralise hexadecane and octacosane are frequently identified as members of the genera *Rhodococcus* and *Pseudomonas* (Aislabie *et al.*, 2012) and sometimes as *Xanthomonas*, *Acinetobacter*

and *Defluvibacter* (Tzintzun-Camacho *et al.*, 2012). The aerobic bacterial AHC degradation pathways have been reviewed (Wentzel *et al.*, 2007). Like other *n*-alkanes, aerobic degradation of hexadecane or octacosane can be initiated by broad specificity Cu-monooxygenases attacking the terminal methyl group to produce primary alcohol or by dioxygenases to form aldehydes through *n*-alkyl hydroperoxides without an alcohol intermediate, respectively. The alcohol is oxidised to the corresponding aldehyde and fatty acid, which are further oxidised by cytoplasmic β–oxidation enzymes to tricarboxylic acid (TCA) (Van Hamme *et al.*, 2003). An alternative sub-terminal oxidation pathway for biodegradation of *n*-alkane with C_3–C_6 and $>C_{24}$ chain lengths to form secondary alcohols and ketones has been reported (Whyte *et al.*, 1998). Paper III investigates the impact of diesel concentration and contact time in soil on indigenous catabolism of hexadecane. Paper XII studies the effects of soil amendments on biodegradation of hexadecane and octacosane by indigenous soil microflora.

1.2.2. Naphthalene

Naphthalene ($C_{10}H_8$) is a PAH with two aromatic rings; it has a molecular weight of 128.2 g mol^{-1} and a log K_{ow} of 3.37 (Table 2). Naphthalene is therefore relatively soluble in water and has a half-life of 80+ days in soil, indicating that it is readily degradable by indigenous microorganisms. Beside its presence in petroleum oil, naphthalene can also be produced naturally by microorganisms associated with termites (Wilcke *et al.*, 2003; Musa Bandowe *et al.*, 2009), and can be produced industrially for use in the production of plastics, dyes, resins, lubricants, and pesticides (Mumtaz *et al.*, 1996).

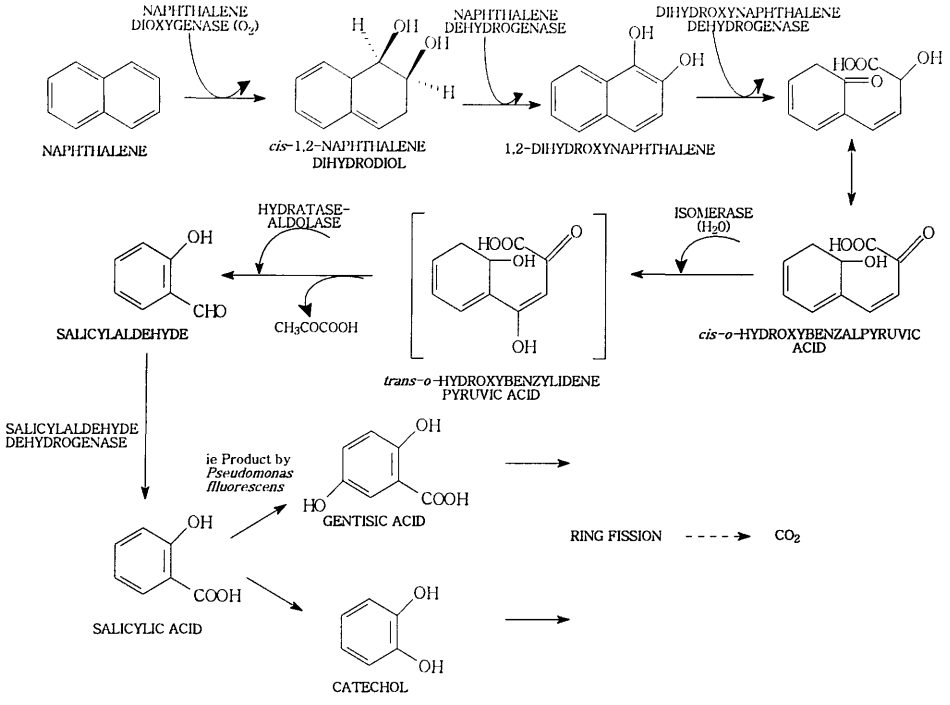
Table 2: Structure and physicochemical properties of priority PAHs (Wild and Jones, 1995; Sverdrup *et al.*, 2002).

PAH	NR[a]	MW[b]	Structure	mp[c]	bp[d]	Sol[e]	K_{ow}[f]
Naphthalene (NC)	2	128.2		79–82	218	32.0	3.37
Acenaphthene (NC)	2	152.2		95	265–275	5.30	3.94
Acenaphthylene (NC)	2	154.2		72–82	96.2	3.93	4.07
Fluorene (NC)	2	166.2		115–116	295	1.85	4.15
Phenanthrene (NC)	3	178.2		99	340	1.29	4.22
Anthracene (NC)	3	178.2		218	340	0.64	4.41
Fluoranthene (NC)	3	202.3		110	~375	0.25	4.74
Pyrene (NC)	4	202.1		156	360	0.14	4.82
Benzo[a]anthracene (C)	4	228.3		158	400–435	0.01	5.25
Chrysene (WC)	4	228.3		255	488	0.002	5.61
Benzo[*b*]fluoranthene (C)	4	252.3		168	–	–	6.11
Benzo[*k*]fluoranthene (C)	4	252.3		215	480	–	6.11
Benzo[*a*]pyrene (SC)	5	252.3		179	496	0.0038	6.04
Dibenz[*a*,*h*]anthracene (C)	5	278.4		273	–	0.0005	6.84
Benzo[*g*,*h*,*i*]perylene (NC)	6	276.4		262	550	0.00026	6.20
Indeno[1,2,3-*c*,*d*]pyrene (C)	6	276.3		163	536	0.062	7.66

[a] NR: number of benzene rings; [b] MW: molecular weight (g mol^{-1}) [c] mp: melting point (°C); [d] bp: boiling point (°C); [e] Sol: aqueous solubility (mg l^{-1}); [f] log K_{ow}: logarithm of the *n*-octanol–water partitioning coefficient. (NC): Non-carcinogenic; (C): Carcinogenic; (WC): Weakly-carcinogenic; (SC): Strongly-carcinogenic

Several microorganisms isolated from environmental samples have been described with the ability to degrade and mineralise naphthalene as a sole source of carbon and

153 energy (Cerniglia, 1992; Juhasz and Naidu, 2000). The indigenous soil microflora in
154 both pristine and contaminated soils have been shown to possess relatively high
155 degradative potentials for naphthalene (Wilcke, 2007; Kumar and Khanna, 2010; Jones
156 *et al.*, 2011). This has been linked to the localisation of most of the aromatic ring-
157 hydroxylating dioxygenases (ARHDs), such as NAH7 plasmid-encoded genes, used in
158 biodegradation of naphthalene and most other LMW-PAHs on conjugative plasmids
159 that can readily transfer horizontally between and within the PAH-degrading
160 populations (Akhmetov *et al.*, 2008). This unique property enhances degradative
161 potentials of soils and promotes rapid adaptation of soil microflora to the presence of
162 these contaminants in the environment (DeBruyn *et al.*, 2011). Naphthalene-degrading
163 bacteria commonly found in contaminated soils belong to the genera *Alcaligenes*,
164 *Burkholderia*, *Mycobacterium*, *Polaromonas*, *Pseudomonas*, *Ralstonia*, *Rhodococcus*,
165 *Sphingomonas*, and *Streptomyces* (Seo *et al.*, 2009). Naphthalene degradative pathways
166 in many bacteria are well characterised (Seo *et al.*, 2009; Baboshin and Golovleva,
167 2012). In general, degradation is initiated through hydroxylation of one of the aromatic
168 rings by the multicomponent ARHD enzyme, naphthalene dioxygenase, to *cis*-(1R,2S)-
169 dihydroxy-1,2-dihydronaphthalene; this is followed by dehydrogenation to 1,2-
170 dihydroxynaphthalene and its subsequent breakdown to salicylate, which is further
171 catabolised via ring fission in the *meta*- or *ortho*-pathways to $CO_2 + H_2O$ (Figure 1).
172 Paper II investigates the impact of diesel concentration and contact time in soil on
173 indigenous catabolism of naphthalene. Paper IX studies the impacts of prescribed fire
174 on biodegradability of naphthalene in diesel oil-amended soil. Paper XII studies the
175 effects of soil amendments on biodegradation of naphthalene by indigenous soil
176 microflora.



177

178 Figure 1: Naphthalene degradative pathways in bacteria (modified from Bamforth and

179 Singleton, 2005).

180

181 *1.2.3. Phenanthrene*

182 Phenanthrene ($C_{14}H_{10}$) is a PAH with three aromatic rings; it has a molecular weight of

183 178.2 g mol^{-1} and a log K_{ow} of 4.22 (Table 2). The presence of phenanthrene in diverse

184 background soils and the global pattern of its distribution indicate that this compound

185 may also be produced naturally (Wilcke, 2007). Relative to other PAHs, phenanthrene

186 is usually found in high concentrations in uncontaminated and petroleum oil-

187 contaminated soils (Juhasz and Naidu, 2000; Agarwal *et al.*, 2009).

188 Factors affecting microbial degradation of phenanthrene in soils have been extensively

189 studied (Bamforth and Singleton, 2005; Seo *et al.*, 2009). Although relatively more

190 persistent in soil than naphthalene, the half-life of phenanthrene ranged widely in soils;

191 14–8157 days in spiked soils or 83–2081 days in sewage sludge-amended soils

192 (Northcott and Jones, 2001), with an average of ≤140 days (Rostami and Juhasz, 2011).

Several microorganisms able to utilise phenanthrene as a sole source of carbon and energy have been isolated and identified to belong to the genera *Acidovorax*, *Arthrobacter*, *Brevibacterium*, *Burkholderia*, *Comamonas*, *Mycobacterium*, *Pseudomonas*, and *Sphingomonas* (Seo *et al.*, 2009).

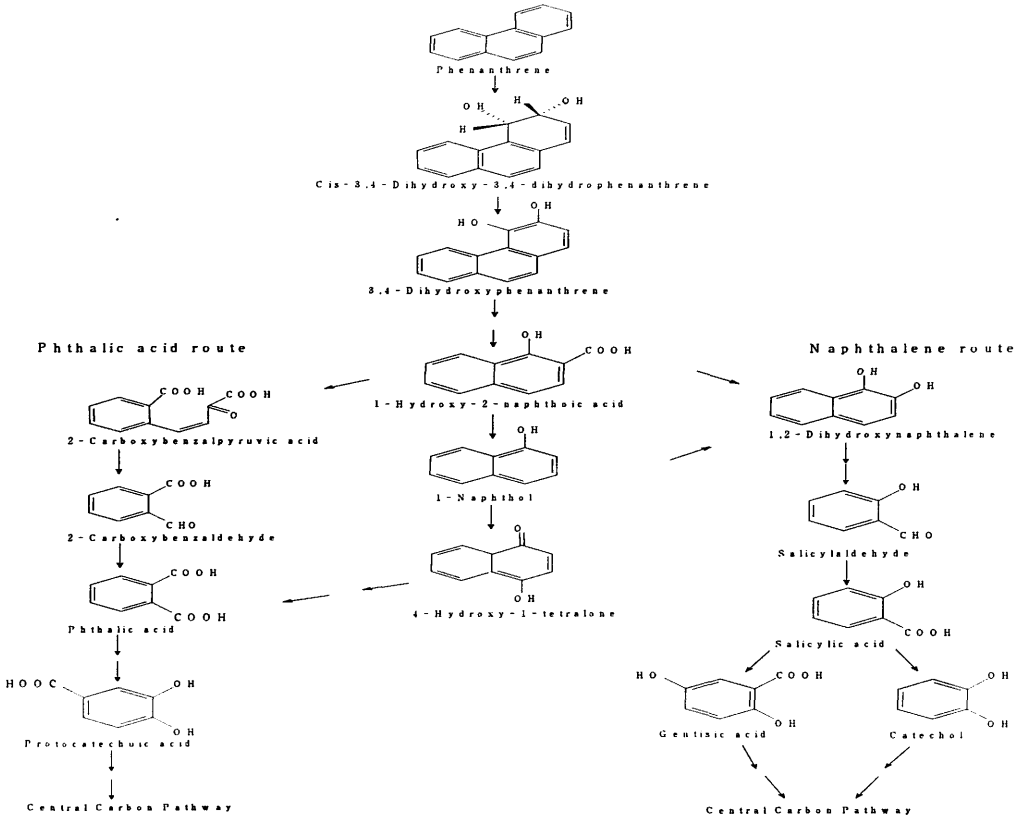


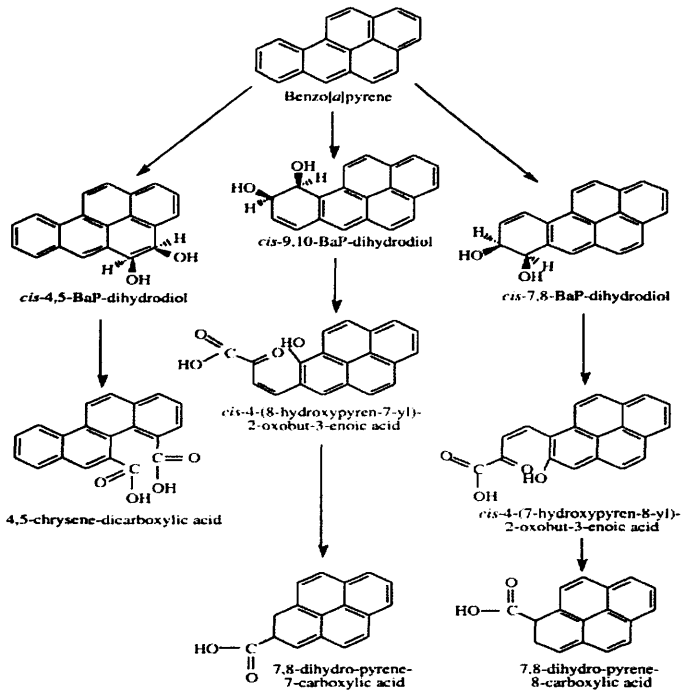
Figure 2: Phenanthrene degradative pathways in bacteria (Seo *et al.*, 2009).

As shown in Figure 2 enzymatic degradation of phenanthrene is usually initiated through the activities of ARHD enzymes on the C_3–C_4 bond to yield *cis*-3,4-dihydroxy-3,4-dihydrophenanthrene, followed by dehydrogenation to 3,4-dihydroxyphenanthrene and further catabolism to 1-hydroxy-2-naphthoic acid. Subsequent cleavage of the ring of the diol via the *meta*- (phthalic route) or *ortho*-pathway (naphthalic route) leads to formation of phthalic acid or salicylic acid, respectively. Because phenanthrene

207 contains bay- and K-regions, formation of an epoxide, which is thought to be
208 carcinogenic, is possible via the oxidative action of cytochrome P_{450} monooxygenases
209 in non-ligninolytic fungi; subsequent catabolism via dehydrogenases and further to ring
210 fission then follows (Samanta *et al.*, 2002; Bamforth and Singleton, 2005).
211 Paper III investigates the impact of diesel concentration and contact time in soil on
212 indigenous catabolism of phenanthrene. Paper V studies the biodegradation of
213 phenanthrene under complex contaminant systems in soils with differing properties.
214 Paper IX studies the impacts of prescribed fire on biodegradability of phenanthrene in
215 diesel oil-amended soil. Paper XII reports the effects of soil amendments on
216 biodegradation of hexadecane and phenanthrene by indigenous soil microflora.
217
218 *1.2.4. Benzo[a]pyrene*
219 Benzo[a]pyrene ($C_{20}H_{12}$) is a PAH with five aromatic rings; it has a molecular weight
220 of 252.3 g mol^{-1} and a log K_{ow} of 6.04 (Table 2). Benzo[a]pyrene has low polarity,
221 solubility and vapour pressure (5.0 x 10^{-7} Pa), as well as a large resonance energy
222 which make the chemical structure thermodynamically stable (Bamforth and Singleton,
223 2005). Aside from its presence in refined petroleum oils, benzo[a]pyrene is generated
224 by incomplete combustion of biomass, and in fossil fuels; there is no commercial
225 production or use of it. Benzo[a]pyrene is one of the most widely studied PAHs due to
226 its ecological and ecotoxicological significance (Juhasz and Naidu, 2000). Studies have
227 demonstrated the genotoxicity of PAHs with at least four rings (Sverdrup *et al.*, 2002;
228 Martin *et al.*, 2005; Hu *et al.*, 2012).
229 Benzo[a]pyrene can cause tumours on experimental animals through various exposure
230 routes, e.g. dermal and oral administration, inhalation, subcutaneous and intramuscular
231 applications (Juhasz and Naidu, 2000). Once it enters into the food chain,

benzo[a]pyrene is transformed to genotoxic metabolites, which can interact with DNA and proteins forming extremely reactive bulky adducts and causing mutagenicity and carcinogenicity in mammals (Juhasz and Naidu, 2000; Hu *et al.*, 2012). A survey of uncontaminated soils in Wales indicate background concentrations of benzo[a]pyrene ranging from 3.5 to 3700 μg kg^{-1} with an average concentration of 16 μg kg^{-1}, in contrast with the overall PAH levels ranging from *ca.* 100 to *ca.* 55000 μg kg^{-1} (Jones *et al.*, 1989b). In another study carried out at Rothamsted Experimental Station in the UK, Jones *et al.* (1989a) reported an increase in benzo[a]pyrene concentration of 20-fold since the 1890s.

Like some other HMW-PAHs, benzo[a]pyrene biodegradation is typically very slow, causing the compound to persist longer in soils than naphthalene and phenanthrene; estimated half-life is ≤2 years (Rostami and Juhasz, 2011). The low levels of benzo[a]pyrene in background soils and the inability of most microorganisms to utilise it as a sole source of carbon and energy are thought to limit its catabolism in soils (Seo *et al.*, 2009). Factors limiting benzo[a]pyrene biodegradability have been reviewed (Juhasz and Naidu, 2000). Relatively few bacteria capable of degrading benzo[a]pyrene have been isolated and described, but those that have belong mainly to the genera *Beijernickia*, *Mycobacterium*, *Pseudomonas*, *Rhodococcus*, *Sphingomonas* and *Stenotrophomonas* (Juhasz and Naidu, 2000); these organisms are not known to mineralise benzo[a]pyrene as pure cultures. A number of fungi including members of the genera *Aspergillus*, *Candida*, *Cunninghamella*, *Nematoloma*, *Neurospora*, *Penicillium*, *Phanerochaete*, *Pleurotus*, *Saccharomyces*, *Syncephalastrum* and *Trametes*, and the algal species *Selenastrum capriconutum* have been reported to biodegrade benzo[a]pyrene in the presence of other growth substrates (Juhasz and Naidu, 2000).



257

258 Figure 3: Proposed benzo[a]pyrene degradative pathway in bacteria (Gibson *et al.*,
259 1975).

260

261 Figure 3 outlines a proposed pathway for the bacterial degradation of benzo[a]pyrene.
262 Similar to the LMW-PAHs, the enzymatic degradation of benzo[a]pyrene in bacteria is
263 initiated by multicomponent dioxygenases to a number of benzo[a]pyrene-dihydrodiols
264 (*cis*-4,5-; *cis*-7,8-; *cis*-9,10-), depending on the bacterial species and growth conditions.
265 The ring cleavage of the hydroxylated compound results in *cis*-4-(-7-hydroxypyren-8-
266 yl)-2-oxobut-3-enoic acid from the *meta* fission (Gibson *et al.*, 1975) or 4,-chrysene-
267 dicarboxylic acid from the *ortho* fission (Schneider *et al.*, 1996).
268 A major part of this thesis is devoted to investigating the effects of diesel concentration
269 on the degradative ability of indigenous soil microflora for benzo[a]pyrene (Papers II
270 and V); the effect of cyclodextrin on enhancing benzo[a]pyrene biodegradation (Papers
271 VI and VII); the contributory effect of bioaccessibility to benzo[a]pyrene

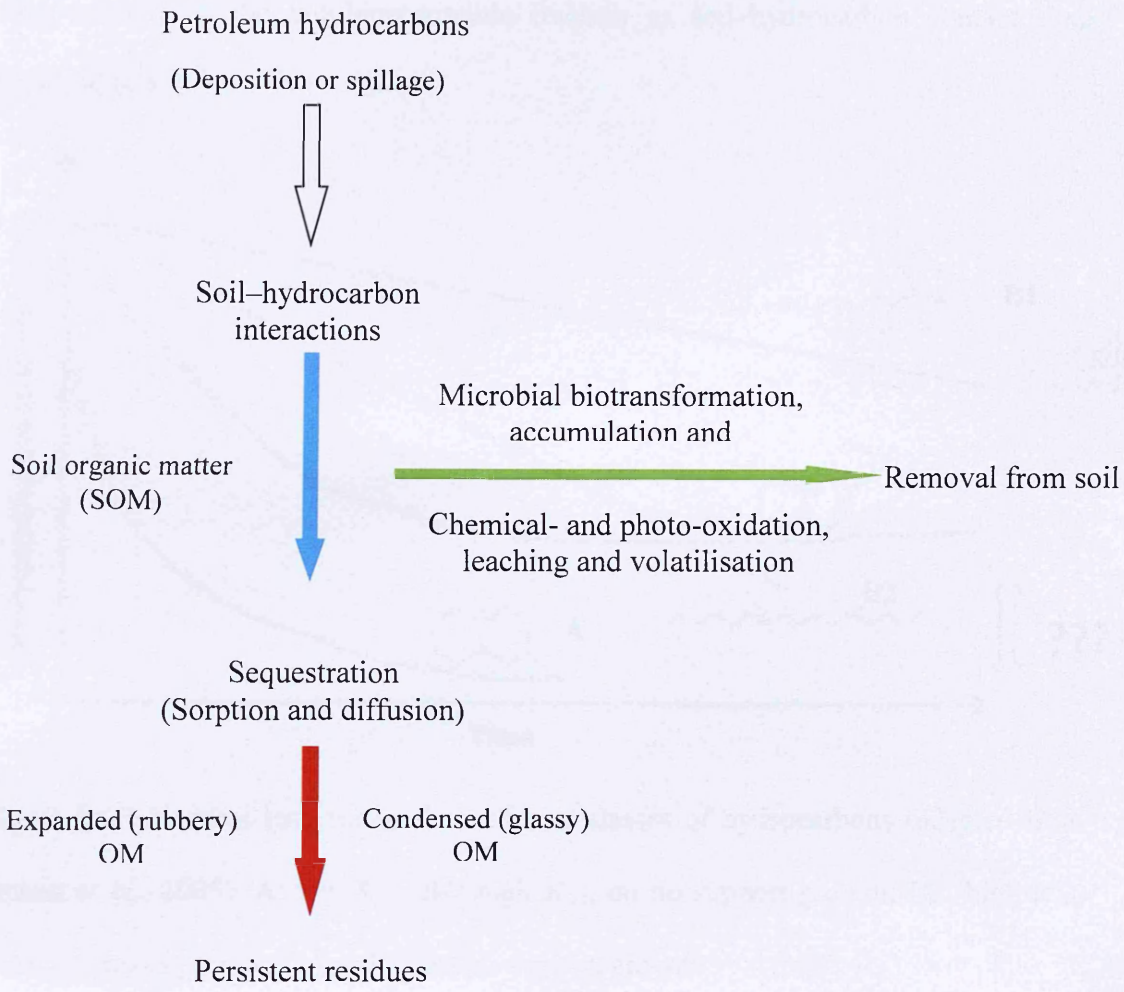
biodegradation (Paper VIII); and the effect of prescribed fire on benzo[a]pyrene biodegradation in diesel oil-amended soil (Paper IX).

1.3. Fate and behaviour and factors affecting hydrocarbon biodegradation in soil

1.3.1. Fate and behaviour of soil-associated hydrocarbons

Spills of petroleum oils readily spread across the soil, depending on the oil viscosity and spill volume, soil texture, vegetation cover and topography; the volatile components evaporate from the surface and the rest dissolve into soil pore, adsorb to soil particulate materials or leach into the subsurface. Microbial degradation begins, as well as chemical or photo-oxidation, which collectively removes a large portion of the oil in soil; sequestration (including sorption and diffusion) of oil into soil also proceeds (Figure 4). Overtime, chemical and biological weathering alter the composition of the spill residues; these processes are dependent on a number of variables including environmental, edaphic and (micro)biological, as well as the physical and chemical properties of individual hydrocarbon (Semple *et al.*, 2003).

The fate and behaviour of hydrocarbons is believed to depend largely on the physicochemical properties of the hydrocarbons and the soil–hydrocarbon–microbes interactions (Hatzinger and Alexander, 1995; Stokes *et al.*, 2005; Stroud *et al.*, 2007a). Figure 5 shows a theoretical loss curve for four different classes of hydrocarbons in soil where microbial action and the physical–chemical interactions of soil with hydrocarbon are occurring simultaneously. In general, hydrocarbons with low K_{ow}, and high aqueous solubility and volatility are more mobile and degradable, resulting in their rapid loss from soil (A); this is typical of naphthalene and other mono-aromatic hydrocarbons.



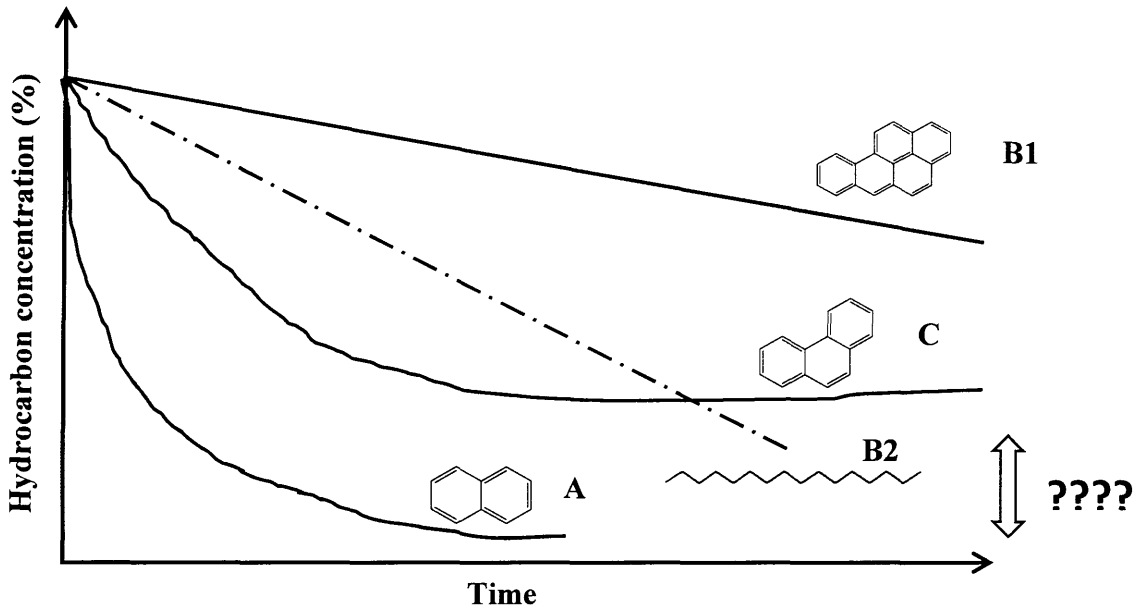
295

296 Figure 4: Fate and behaviour of hydrocarbons in soil (Okere and Semple, 2012).

297

298 Another process, which relates to hydrocarbons with high K_{ow}, and low aqueous
299 solubility and volatility, indicates the very slow degradation of HMW-PAHs that do
300 not readily support microbial growth (B1) or the steady removal of *n*-alkanes that are
301 constitutively biodegradable but not readily bioavailable (B2). The difference in the
302 chemical structure is a significant factor for which AHCs with comparable or higher
303 K_{ow} can be more biodegradable than PAHs. A third process, and which is common to
304 many PAHs, is a biphasic behaviour govern by the counter-balancing of the influences
305 of microbial activity on the readily available/degradable fraction and the sequestration

306 effect of soil on the non-bioaccessible fraction as soil–hydrocarbon contact time
307 increased (C).



308

309 Figure 5: Theoretical loss curves for different classes of hydrocarbons (adapted from
310 Stokes *et al.*, 2005). A: low K_{ow}; B1: high K_{ow}, do no support growth; B2: high K_{ow},
311 readily support growth; C: moderate K_{ow}; support growth.

312

313 The process of sequestration, also termed ageing (Alexander, 1995; Hatzinger and
314 Alexander, 1995), has been demonstrated through various studies to be dependent on a
315 number of factors, including the quality and quantity of soil organic matter (SOM) and
316 mineral components, type and concentration of target hydrocarbon, presence and
317 concentration of co-contaminants, abundance, diversity and degradative ability of
318 microorganisms present, as well as other soil variables like pH, inorganic nutrients,
319 oxygen and moisture content and temperature (Reid *et al.*, 2000). Over time,
320 hydrocarbons become sorbed to organic matter or trapped within micropores in soil;
321 this decreases their bioavailability and makes them resistant to biodegradation,
322 preventing their complete removal from soil (Bosma *et al.*, 1997).

323

324 *1.3.2. Factors affecting biodegradation of hydrocarbons*

325 Once the hydrocarbons enter soil, they can follow a number of different routes, e.g.
326 transformed by photo- and chemical oxidation (Shiaris, 1989), degraded through
327 biological processes, or sequestered to soil particles, as depicted in Figure 4. While
328 some indigenous microorganisms are inherently more adept at degrading specific
329 hydrocarbons, others require time to adapt. Most AHCs, such as hexadecane, possess
330 similar molecular structures to those compounds primarily used by microorganisms in
331 uncontaminated soils, which make them constitutively degradable (Stroud *et al.*,
332 2007b; Wentzel *et al.*, 2007). PAHs, on the other hand, are made up of two or more
333 fused benzene rings which are comparatively resistant to biodegradation and therefore
334 require time for the induction of appropriate catabolic enzymes (Cerniglia, 1992).
335 Inducible degradation is developed in microorganisms through a series of processes,
336 known as adaptation, occurring either individually or in combination: (i) the induction
337 of specific enzymes suitable for the degradation of the hydrocarbon; (ii) increased
338 degradative ability through genetic changes and/or; (iii) selective enrichment of
339 organisms that hold the desired degradative potential (Spain and Van Veld, 1983). In
340 instances where the hydrocarbons, such as benzo[a]pyrene, are typically unsuitable as a
341 sole carbon source, extended time and the presence of other growth substrates and/or
342 microorganisms are required for complete degradation to occur (Dalton *et al.*, 1982).
343 In addition to the abundance and diversity of microorganisms with competent
344 degradative ability for the target hydrocarbons, a number of environmental and edaphic
345 factors also influence, directly or indirectly, the rate and extent of biodegradation.
346 Studies showed that the overall rates of degradation of hydrocarbons are influenced by
347 temperature, pH, oxygen and water content, composition and concentration of

348 inorganic nutrients (Bamforth and Singleton, 2005; Das and Chandran, 2011). In
349 general, optimal degradation of hydrocarbons is thought to occur in soils with a neutral
350 pH, a temperature range of 30–40 °C, a water content of 30–90% of their maximum
351 water holding capacity (WHC), and a C:N:P ratio of 100:10:1.
352 The rate and extent of hydrocarbon degradation in soil have also been shown to depend
353 on the interactions of soil physical–chemical components with the hydrocarbons. The
354 quality and quantity of mineral and organic components of soil, as well as the
355 arrangement of soil micro and nanopores (Nam and Alexander, 1998; Nam *et al.*, 1998)
356 affects, the presence, survival and invariably the overall activity (including degradative
357 ability) of soil microbial community (Macleod and Semple, 2000). Soil–hydrocarbon
358 contact time can also have effects on mineralisation. As contact time increased, the
359 ability of indigenous soil microflora to mineralise PAHs may be enhanced (Macleod
360 and Semple, 2006); this depends on the presence and concentration of co-contaminants
361 (Couling *et al.*, 2010). Bioavailability of hydrocarbon is also affected through various
362 physical–chemical interactions with the soil components over time (Semple *et al.*,
363 2007). Hydrocarbons with a large particle size may expect to be more biodegradable
364 than their smaller size counterparts as they are less likely to become trapped in pore
365 spaces of SOM and mineral components.
366 Results from Papers II, III, V–IX contained in this thesis indicate that the concentration
367 of diesel influenced the rate and extent of mineralisation of naphthalene, phenanthrene
368 and benzo[a]pyrene in soils.
369

1.3.3. Issues of bioavailability/bioaccessibility in biodegradation of AHCs and HMW-PAHs

For biodegradation of hydrocarbons to occurs, two steps are involved: the first, a physical process – the passive uptake of the hydrocarbon molecules by microbial cells, and the second, a biological process – the enzymatic transformation of the compounds in the microbial cells. While the latter is a function of the intrinsic degradative ability of the cells, the former is a function of bioavailability/bioaccessibility of the compound to the cell (Semple *et al.*, 2007). In most cases, mass transfer limitations prevent the full exploitation of the microbial degradative potential (Bosma *et al.*, 1997). The concepts of bioavailability/bioaccessibility and the implications to hydrocarbon biodegradation in soil and sediments have been comprehensively reviewed (Semple *et al.*, 2004; 2007). Bioavailability (i.e., the ability of a compound to be freely transported across the cell membrane for intercellular or available for extracellular metabolism) may be the most important factor in determining the feasibility of bioremediation of PAHs (Semple *et al.*, 2004). Limited bioaccessibility is due to low aqueous solubility of hydrocarbons and their tendency to partition onto soil mineral surfaces or to sorb strongly to the soil organic matrices (Semple *et al.*, 2003). A number of mechanisms are thought to collectively influence bioaccessibility, and different mechanisms predominate in any given situation; although, they are still not fully understood (Semple *et al.*, 2004; Rostami and Juhasz, 2011; Cui *et al.*, 2013).

It is usually assumed that the aqueous phase-dissolved fraction of hydrocarbons is the only one *available* to microorganisms; therefore, biodegradation rate is dependent on the mass transfer of hydrocarbon molecules from solid or soil-bound phase and desorption to the aqueous phase (Pignatello and Xing, 1995). The potentially biodegradable fraction of HOCs in soil, which can be quantified chemically in the

laboratory (i.e., the bioaccessible fraction), consists of the fraction that may readily desorb from soil to and/or is present in the aqueous phase (Semple *et al.*, 2007; Riding *et al.*, 2013). A number of studies have evidenced that microbial uptake of hydrocarbons can also take place directly from soil interphase surfaces (Bogan *et al.*, 2003; Huesemann *et al.*, 2003, 2004). In the case of straight-chain *n*-alkanes like hexadecane, studies to estimate bioaccessibility, and therefore, the extent of mineralisation have showed that the desorbed fraction is always less than that of the microbially-degraded fraction, indicating that the direct uptake of this class of hydrocarbons from the soil inter-phase surfaces without prior desorption to the aqueous phase is also an important contributor to their biodegradation (Huesemann *et al.*, 2003, 2004). For benzo[a]pyrene, the measured bioaccessible fraction appears, in most cases, to be greater than the maximum microbially-degradable fraction (Towell *et al.*, 2011b). Results from Papers VI and VII showed that the sorptive properties (SOM and clay contents) of soil influenced benzo[a]pyrene dissolution rate from soil matrices; however, benzo[a]pyrene mineralisation was negligibly influenced by these properties.

1.3.4. Growth-linked and cometabolic biodegradation – implications for HMW-PAHs

In the laboratory, the kinetics of the biodegradation or mineralisation of target hydrocarbons in soil can be monitored through the conversion of ^{14}C-labelled hydrocarbons to $^{14}CO_2$ and/or intermediate or dead-end ^{14}C-metabolites, which give a representation of the catabolic capabilities of the degrading microorganisms. Typically, hydrocarbon mineralisation occurs in three phases, and is usually represented by a sigmoidal growth curve: the lag phase where the microbes are adapting and reproducing; the rapid growth or the exponential phase where the catabolic activity of the microbes is at its peak and mineralisation is rapidly occurring; and the plateau or

420 the stationary phase where mineralisation has reached its maximum extent and $^{14}CO_2$
421 production ceases. Semple *et al.* (2006) established that the maximum extent of
422 microbial mineralisation of a contaminant is directly proportional to the
423 bioaccessibility of the contaminant in soil and not the result of factors limiting the
424 catabolic activity of the microbes, such as lack of nutrients or mortality of the microbes
425 themselves. This appears to be largely true for hydrocarbons that can serve as growth
426 substrates for the degrading microorganisms. By contrast, the findings of research on
427 benzo[a]pyrene reported in this thesis (Papers II, V, VI, VII and VIII) indicate that
428 bioaccessibility plays a lesser role than the abundance and degradative ability of
429 degrading microbial community in benzo[a]pyrene mineralisation.
430 The inability of benzo[a]pyrene to readily serve as substrate for microbial growth
431 means its degradation depends on fortuitous catabolic activity of the degrading
432 microorganisms (Kanaly and Harayama, 2000; Kanaly *et al.*, 2001; Kanaly and
433 Watanabe, 2004). This fortuitous action of microorganisms has been described as
434 cometabolism (Dalton *et al.*, 1982; Slater *et al.*, 1982). Cometabolism is the
435 transformation of a non-growth substrate in the obligate presence of a growth substrate
436 or another transformable compound (Dalton *et al.*, 1982). Recently, an update of the
437 available literature on cometabolic degradation of HOCs has been reviewed (Nzila,
438 2013). Cometabolism appears to play an important role when assessing the degradation
439 capacities of the HMW-PAHs when their LMW-PAH counterparts are available as
440 primary substrate to supply carbon and energy that support microbial growth, allowing
441 the degradation of non-growth substrate (Juhasz and Naidu, 2000). The co-metabolite
442 may also induce production of catabolic enzymes that can catalyse the transformation
443 of the non-growth substrate (Gibson *et al.*, 1975; Robertson and Alexander, 1994).
444

1.3.5. Effect of co-contaminant concentrations on biodegradability of target hydrocarbons

An important factor that may impact on hydrocarbon biodegradation in field contaminated soils is the presence of co-contaminants; it is very rare to find a situation where contamination arises from the presence of a single chemical. Biodegradation of a target hydrocarbon may proceed at different rates and to different extents due to various biological, chemical and/or physical limitations or changes induced by the presence of co-contaminants (Bouchez *et al.*, 1995; Ghoshal and Luthy, 1996). These changes may affect the level of extant microbial activity as well as the extent of bioavailability, and the subsequent biodegradation of the target hydrocarbon (Hughes *et al.*, 1997). Findings from previous studies suggested that that the development of PAH catabolism is enhanced or repressed in the presence of co-contaminants (Bauer and Capone, 1988; Efroymson and Alexander, 1994; Bouchez *et al.*, 1995; Kanaly *et al.*, 1997; Bouchez *et al.*, 1999; Kanaly *et al.*, 2001; Swindell and Reid, 2006; Couling *et al.*, 2010). Collectively, these studies attributed the antagonistic and/or synergistic interactions between microbial populations as well as the competitive and/or simultaneous degradation of co-contaminants to the types and concentrations of the co-contaminants, the kinds of microorganisms present and their catabolic preferences. Most of these studies, however, have been conducted using binary or tertiary mixtures of hydrocarbons, with only a few conducted in soils containing complex mixtures of hydrocarbons, which reflect environmental scenarios and are more representative of soils polluted with diesel, coal tar or creosote. Papers II, III, VI and IX contained in this thesis address the effects of increasing concentrations of diesel on the development of hydrocarbon degradative ability in soil. A direct quantitative comparison is generally

469 lacking which considers variability in soil types. Papers V, VII and VIII comparatively
470 assess PAH biodegradation in different soil types.
471
472 *1.3.6. Impact of prescribed fire on remediation of petroleum oil-contaminated soils*
473 In practice, due to certain peculiar challenges, such as accessibility constraints to sites,
474 the prohibitive cost and/or ineffectiveness of adapting alternative options to specific
475 site conditions, as well as the regulatory requirements for immediate mandatory
476 actions, *in situ* prescribed fire is often permitted as a first-line remediation operation to
477 remove a large portion of the oil contaminants from the top soil or sediment surface. In
478 the Gulf of Mexico and many sensitive coastal areas of North America, *in-situ*
479 prescribed fire has been used, subject to regulatory approvals (Martin Jr *et al.*, 2003), to
480 remediate oil-impacted wetlands and marshes (Lin *et al.*, 2002; Lindau *et al.*, 2003; Lin
481 *et al.*, 2005). This operation has been used also in inland and upland environments to
482 prevent spreading of oil to sensitive sites or larger areas, or to reduce the generation of
483 oily wastes, especially where transportation or disposal options are limited (Zengel *et*
484 *al.*, 2003).
485 Much of the understanding of the impact of prescribed fires for oil spill remediation are
486 based on work in the open sea, wetlands and other coastal land environments (Fritz,
487 2003; Ko and Day, 2004), with research efforts focussed toward general ecological
488 function and structure including species composition and density, above- and below-
489 ground productivity, vegetation and soil resiliency, soil physics and chemistry, soil
490 residual oil, and organic matter decomposition (Baustian *et al.*, 2010). The impact of
491 prescribed fires (and sometimes accidental or deliberate act of sabotage) on the
492 indigenous microbial community in petroleum oil-contaminated upland environment
493 has rarely been investigated (Zengel *et al.*, 2003), and to date, there is no study of the

494 impact on degradative ability of the indigenous soil microflora to catabolise the
495 residual oil. As a credit to its novelty, this thesis made a modest attempt to investigate
496 the impact of prescribed fire on the degradative ability of indigenous soil
497 microorganisms to mineralise target PAHs (Paper IX).
498
499 **1.4. A brief remark on diesel oil**
500 In all of the studies presented in this thesis (except for Paper XII), diesel oil was used.
501 During crude petroleum refining diesel oil is derived from the middle-distillate, gas-oil
502 fraction and is composed of a complex mixture of normal, branched, and cyclic alkanes
503 (60 to >90% by volume; hydrocarbon chain length usually between C_9 and C_{30});
504 aromatic compounds, especially alkylbenzenes (5–40% by volume; and small amounts
505 of alkenes (0–10% by volume) (International Program on Chemical Safety, 1996).
506 Benzene, toluene, ethylbenzene, and xylenes (BTEX) and PAHs, especially
507 naphthalene and its methyl-substituted derivatives, may be present at levels of ppm in
508 diesel oil. At room temperature, diesel fuels are generally moderately volatile, slightly
509 viscous, flammable, brown liquids with a kerosene-like odour. Important variables are
510 ignition behaviour (expressed in terms of octane number), density, viscosity, and
511 sulphur content. The boiling ranges are usually between 140 and 385 °C (> 588 °C for
512 marine diesel oil); at 20 °C, the density is 0.87–1.0 g cm^{-3} and the aqueous solubility is
513 0.2–5 mg l^{-1}. The sulphur content of diesel fuels depends on the source of crude
514 petroleum and the refinery process; it is regulated by law in a number of countries and
515 is usually between 0.05 and 0.5% w/w. Additives are used to influence the flow,
516 storage, and combustion of diesel fuels, to differentiate products, and to meet
517 trademark specifications; the specifications of commercial diesel fuels differ
518 considerably in different countries.

519 In the last three decades, global demand for crude oil, and in particular distillate fuel
520 oils (including diesel and gasoline), has increased steadily. World crude oil
521 consumption (in million barrel d^{-1}) increased from 57.4 in 1985 to 70.1 by 1995 and to
522 87.4 by 2010. Distillate fuel oils consumption (in mb d^{-1}) increased from 7.9 in 1985 to
523 18.2 by 1995 and to 25.0 by 2010. Diesel fuels consumption is projected to increase to
524 28.7 mb d^{-1} in 2015 and to 35.0 mb d^{-1} by 2030 (OPEC, 2011). By region, distillate
525 fuel oils consumption increased from 3.5 in 1985 to 4.8 by 2010 for North America;
526 from 3.3 in 1985 to 7.2 by 2010 for Europe; and from 0.5 in 1985 to 1.2 by 2010 for
527 Africa (OPEC, 2011). Diesel oils are mostly used as transportation fuels; they are also
528 used in stationary engines and in boilers, e.g. reciprocating engines, gas turbines,
529 pipeline pumps, gas compressors, steam processing units in electric power plants,
530 burner installations, and industrial space and water heating facilities. The quality and
531 composition of diesel oil influence the emissions of pollutants from diesel engines
532 considerably (International Program on Chemical Safety, 1996). The more volatile
533 fuels, with low viscosity, are usually required for high-speed engines and the heavier
534 grades for railroad and ship diesel engines. Although the consumption of diesel fuel in
535 passenger cars powered by diesel engines is low (1–2%) and declining in North
536 America, the rate is high (10–25%) and increasing in Europe and parts of Asia,
537 including Japan and China.
538 As diesel oils are complex mixtures, there is no specific analytical method, and the
539 analytical techniques used in most environmental assessments are suitable only for
540 measuring the total petroleum hydrocarbons (International Program on Chemical
541 Safety, 1996; Wang *et al.*, 1999). In general, the methods consist of preliminary solvent
542 extraction, a clean-up procedure to remove naturally occurring hydrocarbons, and
543 subsequent detection by gravimetry, infrared spectroscopy or gas chromatography

544 (Boehm *et al.*, 1998; Wang *et al.*, 1999; Wilcke, 2007; Al-Mutairi *et al.*, 2008). Neither
545 the gravimetric nor the infrared technique provides useful qualitative or quantitative
546 information on contaminants and can thus be used only for screening. Standard
547 procedure for analysing environmental samples involves gas chromatography
548 combined with detection techniques, such as flame ionisation (GC-FID) or mass
549 spectrometry (GC-MS); however, many other methods are available for the analysis of
550 individual hydrocarbons in diesel fuels (Wang *et al.*, 1999; Wang and Fingas, 2003).
551 The movement of diesel oil through soil is thought to be governed by its kinematic
552 viscosity as well as the moisture content and nature of soil; although there are no
553 experimental data to support this. The log K_{ow} of diesel fuels is 3.3–7.06, suggesting a
554 high potential for bioaccumulation; however laboratory data on bioaccumulation of
555 diesel oil in living tissues are scanty (Salanitro *et al.*, 1997; Dorn *et al.*, 1998).
556 Meanwhile, there are many laboratory-based and field-scale studies that evidenced
557 bioaccumulation and biomagnification of individual constituents of diesel oils,
558 especially PAHs, in animal and plant tissues (Matscheko *et al.*, 2002; Parrish *et al.*,
559 2006; Khan *et al.*, 2008; Heijden and Jonker, 2009; Gomez-Eyles *et al.*, 2010; Bielská
560 *et al.*, 2013). Most of the individual constituents are inherently biodegradable, to
561 varying extents and at different rates. The *n*-alkanes, *n*-alkylaromatic, and simple
562 aromatic molecules in the C_{10}–C_{22} range are the most readily degradable; smaller
563 molecules are generally rapidly metabolised. Long-chain *n*-alkanes are more slowly
564 degraded owing to their hydrophobicity and because they are viscous or solid at
565 ambient temperatures while branched alkanes and cycloalkanes are relatively resistant
566 to biological attack, and PAHs are resistant.
567
568

2. Aims and objectives of the thesis

Since the industrial revolution in the mid-18th century, and the later engineering improvements made to the internal combustion engine in the early 20th century, there has been tremendous increase in the production, and usage of petroleum oils as a principal source of energy to drive heavy machineries with attendant increase in the pollution of the environment. Globally, an estimated 1.7–8.8 million tons of petroleum products are released annually into aquatic and soil environments (Leahy and Colwell, 1990). In the UK, hydrocarbon pollution accounts for over 15% of all pollution incidents, averaging nine incidents per day in 2005 (Stroud *et al.*, 2007b). Because of the enormous socio-economic, ecological and ecotoxicological (including human health) significance of petroleum hydrocarbon pollution, research into detoxification of polluted environments remains very vital to sustainable development. The need is even greater in the soil environment because of the complexity in the interactions between biological, chemical and physical elements that determine the fate of hydrocarbon pollutants and the high variability of the effects in soil. Biodegradation is the principal means of hydrocarbons removal from soil, hence, the study of the different aspects of the biodegradability of hydrocarbons in soils contaminated with petroleum oils is of immense importance.

Although there is a large collection in the literature of studies focused on factors affecting biodegradability of hydrocarbons in soils, only a handful of these investigated the impact of co-contaminant concentration under complex hydrocarbon-mixture systems to reflect environmental scenarios, which are more representative of soils polluted with petroleum oils. To date, where studies have been conducted in complex hydrocarbons-polluted soils, none has directly and quantitatively compared the influence of variability in soil types. Obviously, it may not always be accurate to

extrapolate findings from studies in one soil type to soils with different properties because of the wide variability in soil properties and the dependence of hydrocarbon biodegradation on these properties. Further, there is no information in the literature on the development of indigenous degradative ability for benzo[a]pyrene along concentration-gradient in soils polluted with complex hydrocarbon mixtures in soils. Such investigations, however, have important implications because contaminant concentrations varied widely at contaminated sites even on a millimetre/centimetre-scale. Likewise, the impact of diesel concentration on the ability of soil microorganisms to degrade AHCs has rarely been studied despite that petroleum oils contain up to 90% by volume of this group of hydrocarbons; oil pollution in the UK is dominated by diesel (Stroud *et al.*, 2007b). Knowledge of the effects of exposure concentration and history under complex co-contaminants systems is of importance in designing and evaluating bespoke strategies for contaminated land clean-up.

Therefore, the aims of this thesis were to:

i. assess the effect(s) of increasing diesel oil concentration on the development of indigenous degradative ability for hydrocarbons in soils

ii. assess the effect(s) of increasing diesel oil concentration on the overall metabolic responses and efficiency of indigenous microorganisms in soils

iii. assess the effect(s) of cyclodextrin presence and concentration, diesel presence and concentration, and soil physicochemical characteristics on the bioaccessibility and the biodegradability of the potent carcinogen benzo[a]pyrene in soils

iv. evaluate the effect(s) of various amendments (root exudates) and treatments (in this case prescribed fire) on hydrocarbon biodegradation in soil

3. Précis of results from Papers I–XII

Paper I: In this paper, the applicability and limitations of solubility enhancement agents (SEAs), such as surfactants and cyclodextrins, in bioremediation of PAH-contaminated soils and sediments is reviewed with particular attention directed at the HMW-PAH, benzo[a]pyrene. As specific information on SEA-assisted biodegradation of benzo[a]pyrene is scanty, a synthesis of the relevant studies on LMW-PAHs and other HMW-PAHs cited in the literature is used to draw up inferences of the effects of SEAs on the bioaccessibility and biodegradation of benzo[a]pyrene. The implications of SEA-assisted benzo[a]pyrene biodegradation for contaminated land risk assessment and remediation management are highlighted. This review also draws from the findings in some of the studies (Papers II, V–IX) reported in this thesis.

Paper II: This study compares the impact of increasing diesel concentration (applied as log increments from 0 to 10,000 mg kg^{-1}) on the development of indigenous catabolic activity towards two model PAHs, naphthalene and benzo[a]pyrene. Naphthalene catabolic activity was enhanced, increasing as diesel concentration increased in soil up to 1000 mg kg^{-1}. Whereas, there were negligible effects in soils amended to lower diesel concentrations (1–100 mg kg^{-1}), benzo[a]pyrene catabolic activity was significantly enhanced in soil amended to 1000 mg kg^{-1}. Although initially enhanced, the later progressive repression of the indigenous catabolic activity for naphthalene and benzo[a]pyrene suggests the effect of rapid nutrient depletion and increased toxicity in soil amended to 10,000 mg kg^{-1}. This study is novel in that, currently, there is no information in the literature on the development of indigenous catabolic activity towards HMW-PAHs like, benzo[a]pyrene, along a concentration-gradient of complex co-contaminant mixtures in soil. The findings have both spatial

644 and temporal implications, and highlight the need to consider the potential variability
645 in indigenous catabolic activity when designing bespoke remediation strategies since it
646 has been found that contaminant concentrations varied widely, even on a
647 millimetre/centimetre-scale in polluted soils.
648
649 **Paper III**: In support of the findings of Paper II, Paper III compares the impact of
650 diesel concentrations (applied as log increments from 0 to 10,000 mg kg^{-1}) on the
651 indigenous catabolism of a model PAH, ^{14}C-phenanthrene and a model AHC, ^{14}C-*n*-
652 hexadacene. Phenanthrene catabolic activity was significantly enhanced for the first 42
653 d of soil–diesel contact, being higher at concentrations of 10–100 mg kg^{-1} than at 1000
654 mg kg^{-1}; but was persistently repressed ($P < 0.05$) at 10,000 mg kg^{-1}. Hexadecane
655 catabolic activity was marginally repressed along diesel concentration-gradient for the
656 first 21 d and minimally enhanced thereafter. The findings further highlight the
657 constitutive nature of the enzymatic pathways usually deployed for *n*-alkane
658 biodegradation as well as the co-contaminant concentration-dependence of the
659 inducible enzymatic systems required for PAH biodegradation. The potential
660 variability in the development of indigenous catabolic activity due to the spatial
661 heterogeneity in contaminant levels should be taken into account when assessing
662 natural attenuation sites or designing bespoke strategies for enhanced bioremediation.
663
664 **Paper IV**: In addition to investigating the impact of diesel concentration on
665 hydrocarbon catabolic activity of the indigenous microbial community of a pasture soil
666 (Papers II and III), the effect on the overall metabolic responses of the extant soil
667 microflora to fresh input of ^{14}C-glucose is also studied (Paper IV). Whereas, low diesel
668 concentrations (1–100 mg kg^{-1}) had negligible effects, higher concentrations (1000–

669 10,000 mg kg^{-1}) shifted the pattern of ^{14}C-glucose utilisation with greater allocation to
670 ^{14}C-biomass as soil–diesel contact time increased. This was complemented by the
671 relatively greater increases in the abundance of phenanthrene- and octacosane-
672 degrading bacteria following ^{14}C-glucose mineralisation in the 1000–10,000 mg kg^{-1}
673 soils. It is suggested that the actively-growing hydrocarbon-degrading microorganisms
674 in the highly-polluted soils are more likely to preferentially metabolise the easier-to-
675 degrade and higher energy-yielding carbon substrates for biosynthesis rather than for
676 respiration or maintenance requirements.
677
678 **Paper V**: An important factor that may impact on PAH biodegradation, in field
679 contaminated soil, is the presence of other hydrophobic organic contaminants (HOCs),
680 in that it is very rare to find a situation where contamination arises from the presence of
681 a single chemical. In soils contaminated with complex HOC mixtures, PAH catabolic
682 activity may be enhanced or repressed depending on the co-contaminant concentration
683 and contact time in soil; however, a direct quantitative comparison is generally lacking
684 which considers variability in soil type and the physicochemical properties of target
685 contaminants. Paper V advances the studies reported in Papers II and III by
686 investigating four other soils with contrasting physicochemical and microbiological
687 characteristics in order to gain further understanding of the impact of diesel
688 concentration. Collectively, results indicate that due to the inherently high degradative
689 potentials for phenanthrene in the soils, diesel, in the range of 500–5000 mg kg^{-1}, had
690 minimal effect on the extent of phenanthrene catabolism. Meanwhile, the presence of
691 diesel and the soil-contact time were critical for benzo[a]pyrene catabolism in most
692 soils. The findings emphasise that more than for phenanthrene, the development of
693 benzo[a]pyrene catabolism varies widely with soil type, and depends on the

694 concentration of co-contaminants present. Of particular interest is the mineralisation of
695 benzo[a]pyrene in the Nether-Kellet soil, which was found to occur to much higher
696 levels than previously reported in any other background soils. This paper is also the
697 first to report extensive benzo[a]pyrene mineralisation in an Antarctic soil, naturally or
698 artificially polluted; this is particularly remarkable, in that to date, mineralisation of
699 mainly *n*-alkanes and two- to four-ringed PAHs has been reported in soils from this
700 region. Taking into account the stringent restrictions imposed by the 1991 Protocol on
701 Environmental Protection to the Antarctic Treaty on Party States to import "exotic"
702 microorganisms to the Antarctic region, this study shows that native soil microflora, if
703 enriched, can be successfully used to degrade HMW-PAHs in polluted Antarctic soils.
704
705 **Paper VI**: It is well-known that low aqueous solubility and poor microbial degradative
706 activity limit the removal of benzo[a]pyrene from soil. This paper reports the effects of
707 hydroxypropyl-β-cyclodextrin (HP-β-CD) concentrations (0, 12.5, 25 and 50 mM) and
708 repeated exposures over 150 d to diesel oil (1x500, 1x5000, 2x250, 2x2500, 5x100 and
709 5x1000 $mg_{oil\text{-}C}\ kg^{-1}{}_{soil}$) on benzo[a]pyrene mineralisation in soil. HP-β-CD has the
710 ability to massively improve the bioaccessibility of erstwhile sparingly soluble HOCs
711 like benzo[a]pyrene. Consistent with the findings of Papers II and V, the poor
712 indigenous catabolic potential in the background soil was significantly enhanced by
713 pre-exposure to diesel. Results show that to sustain an enhanced benzo[a]pyrene
714 catabolic activity in soil, there is need for constant supply, at the right concentrations,
715 of compounds that can support microbial growth. The main highlight of this study is
716 the inability of HP-β-CD, with increasing concentration, to enhance benzo[a]pyrene
717 mineralisation despite significantly increasing the apparent aqueous dissolution of
718 benzo[a]pyrene from soil matrices. The finding that the induction of catabolically-

competent microorganisms has a greater effect than enhanced bioavailability to expedite benzo[a]pyrene mineralisation in soil is important in predicting the environmental fate of the contaminant, and designing bespoke remediation strategies for soils chronically exposed to petroleum-derived oils.

Paper VII: As a sequel to Paper VI, and because successful implementation of *in situ* bioremediation of PAHs is contingent upon a good understanding of the effects of a variety of soil biotic and abiotic factors on contaminant fate, Paper VII reports HP-β-CD-enhanced bioaccessibility and biodegradation of benzo[a]pyrene in four contrasting soils. The selection of the soils (organic carbon ranged from 0.25 to 20% and clay contents ranged from undetectable to 42%) provides a good basis for comparing the influence of soil–PAH interactions on HP-β-CD-enhanced benzo[a]pyrene bioaccessibility and biodegradation. Soil physical-chemical characteristics affect the extent of benzo[a]pyrene solubilisation by HP-β-CD but have negligible influence on the extent of benzo[a]pyrene mineralisation. Consistent with previous findings, diesel acted to support increase in the populations of indigenous benzo[a]pyrene-degrading microorganisms and as a primary substrate for the induction of benzo[a]pyrene cometabolism. A major implication of the finding that the presence of HP-β-CD reduced the extent of ^{14}C-benzo[a]pyrene mineralisation in three of the soils, is that, in practice, it may be futile or, in certain circumstances, counterproductive to improve benzo[a]pyrene solubilisation in soils lacking robust degradative ability. If HP-β-CD–benzo[a]pyrene inclusion complexes are very stable, as predicted by the stabilisation constants found in the literature, its use in bioremediation of benzo[a]pyrene-contaminated soils and sediments may present greater risks to underground aquifers and increased toxicity to other environmental receptors, and may also result in

744 additional cost for bioremediation. To the authors' knowledge, Papers VI and VII are
745 the first set of studies in the literature on cyclodextrin-enhanced benzo[a]pyrene
746 degradation by indigenous soil microorganisms.
747
748 **Paper VIII**: To further expound the findings of Papers VI and VII, and as an offshoot
749 of the experiments presented in Paper V, Paper VIII reports that there is no relationship
750 between the endpoint mineralisation rates and the amounts of benzo[a]pyrene residues
751 remaining bioaccessible after mineralisation has plateaued. The wide range in the
752 organic carbon (0.25 to 20%) and clay contents (undetectable to 42%) of the soils
753 investigated provides a good basis to assess the influence of soil–PAH interactions on
754 bioaccessibility and mineralisation rates of benzo[a]pyrene in soil slurries. The study
755 highlights that, unlike other LMW-PAHs that can readily serve as microbial growth-
756 substrates, the progress and/or termination of benzo[a]pyrene mineralisation is
757 influenced more by factors limiting microbial degradative activity and far less by the
758 contaminant bioavailability. The finding of Paper VIII is in agreement with those of
759 Papers VI and VII, and may explain further some of the findings of Papers II and V.
760
761 **Paper IX**: In practice, due to certain site constraints, cost-effectiveness of alternative
762 options and/or regulatory requirements, prescribed fire may be permitted as a first-line
763 remediation option for large petroleum oil spills; although, its impact on the
764 degradative ability of indigenous soil microbial community has rarely been
765 investigated. The literature on fire ecology and effects is dominated by studies of
766 prescribed fires as a natural resource and land-use management strategy with little
767 attention paid to the practice as contaminated land remediation operation. Although
768 preliminary in scope, to the authors' knowledge Paper IX presents the first effort aimed

769 at assessing the impact of prescribed fire on the PAH degradative ability of indigenous
770 soil microflora following short- and long-term post-treatments. Consistent with the
771 general opinion from a large collection of both laboratory-based and field-scale studies
772 of prescribed fires in forest and wetland ecosystems, the results of this study indicate
773 that low-severity prescribed fire did not significantly affect, in the long-term, the
774 abundance and the PAH degradative ability of the extant soil microflora while it
775 substantially reduced the PAH burden in soil. In the context of BATNEEC and BPEO,
776 and to achieve remediation goals of rapid reduction in the contaminant burden and/or
777 minimal level of residual contaminants in soil, this study suggests that prescribed fire
778 (with low intensity and short time) is a practicable first-line remediation option.
779
780 **Paper X**: One of the intentions of the experiments presented in Paper X is to answer a
781 query on the use of a "fixed" extraction efficiency factor (i.e. k_{EC}) to convert C-flush to
782 biomass-C, as raised in Paper IV. The quantification of soil microbial biomass-C is
783 essential in assessing nutrient fate and transformations, predicting energy flux and
784 understanding ecosystem processes and functioning in soil; however, to date, there is
785 no direct means of quantifying biomass-C accurately. The chloroform fumigation-
786 extraction (FE) technique, being the most widely used for biomass-C quantification in
787 soils, is thought to be limited by certain interfering factors (moisture content, pH,
788 SOM) or interacting compounds (e.g., black carbon). Unfortunately, most studies on
789 contaminated soils have used the common k_{EC} value of 0.37 or 0.45 derived from
790 studies of agricultural soils without taking into cognisance the interference that
791 contaminants may have on the extraction process and the potential error that could
792 result from this. Remarkably, results from soils with differing physicochemical and
793 microbiological characteristics show that the presence of high diesel concentrations

(1000–5000 mg kg^{-1}) interfered with the extraction process; k_{EC} values were significantly smaller, resulting in overestimation of ^{14}C-biomass in the highly-amended soils. Although, the study is not proposing another "ideal" k_{EC} value, it is advised that, except on the basis of comparison only, an *in-situ* derived k_{EC} value is more appropriate to quantify biomass-C in soils contaminated with petroleum-derived oils.

Paper XI: In distinction to the experiments reported in Paper IV, which measure microbial metabolic responses to fresh input of ^{14}C-glucose at different soil–diesel contact times, the experiments presented in Paper XI examine the influence of diesel concentration and soil texture on the microbial utilisation efficiency and short-term turnover of the readily-available and labile carbon substrate present at low concentration in soil. There is substantial evidence in the literature that soil characteristics, such as clay content and mineralogical composition, can influence the decomposition and stability of labile carbon substrates in soil. However, the influence of xenobiotic organic contaminants like, petroleum hydrocarbons, on the turnover of labile carbon substrates in soil is rarely investigated. To the authors' knowledge, this is the first report of the combined influence of petroleum hydrocarbon contaminants and soil texture on the microbial utilisation efficiency and short-term turnover of labile carbon substrate in soil. Similar to previous findings in heavy metal polluted soils, increased $^{14}CO_2$ respiration accompanied by decreased ^{14}C-biomass formation (i.e., increased $q^{14}CO_2$) as diesel concentration increased indicate the greater "stressed" metabolic states of the extant soil microorganisms. Collectively, the results suggest that the indigenous microorganisms in diesel-contaminated soil expend more energy for maintenance requirements and were less efficient in the utilisation of labile substrates for biomass synthesis; but the effect was less obvious as SOM and clay contents

819 increased in soil. The turnover of labile carbon was faster as diesel concentration
820 increased and decreased as SOM and clay contents increased in soil. This is
821 attributable, in part, to changes in the microbial community structure and, in part, to the
822 effect on clay stabilisation capacity as a result of larger amounts of the oil contaminants
823 being adsorbed to sites on clay materials. This study also further supports the assertion
824 that clay plays an important role in the initial mineralisation and the later
825 decomposition of labile carbon substrate through stabilisation and protection of the
826 microorganisms.
827
828 **Appendix**
829 **Paper XII**: The effects of rhizosphere soil and root tissue amendments are compared
830 by measuring indigenous catabolism of ^{14}C-labelled naphthalene, phenanthrene,
831 hexadecane and octacosane in freshly–spiked and 28-d–aged soils. The rhizosphere soil
832 and root tissues of reed canary grass (*Phalaris arundinacea*), channel grass (*Vallisneria*
833 *spiralis*), blackberry (*Rubus fructicosus*) and goat willow (*Salix caprea*) were collected
834 from a decommissioned petroleum refinery site. Amendment of a pristine grassland
835 soil with the rhizosphere soil or root tissues (5% wet weight basis) significantly
836 enhanced the catabolism of the PAHs but not the *n*-alkanes; pre-exposure increased the
837 ability of indigenous microorganisms to catabolise the PAHs but not the *n*-alkanes.
838 This study highlights that adapted rhizospheric microorganisms or root materials from
839 contaminated sites have beneficial effects as bioaugments or biostimulants,
840 respectively, in freshly PAH-contaminated soils. The findings of this study contribute
841 to the literature on plant-related bioremediation of PAHs.
842

4. General discussion and conclusions

In recent years, one of the burning issues at various environmental discourses, from local to national to global, is the increasing threat posed by pollution to sustainable development. This is as a result of the persistence of these pollutants in the environment and their accumulation in living tissues, which is further compounded by their recalcitrance to biodegradation. Current policies and legislation drives in Europe and North America are towards a continuous reduction in the amount of persistent organic pollutants (POPs) emitted into the environment from primary sources like the industries and motor vehicles, and the decontamination of legacy polluted sites (Latawiec *et al.*, 2010; Riding *et al.*, 2013). Soil contamination with petroleum hydrocarbons can cause distinct changes to soil microbial community structure and functional diversity (Aislabie *et al.*, 2004), which sometimes may result in extensive deterioration of the overall soil ecosystem (Ko and Day, 2004). The distribution pattern of PAHs indicates that soils, acting as sinks as well as secondary sources, make significant contribution to the global inventory of these compounds (Wilcke, 2007). In the studies (Papers II–XI) contained in this thesis, PAHs were found in agricultural and pasture soils from UK, and even in a soil from the remote Antarctic region, which were considered as pristine; this indicates a global distribution of these POPs. Findings also support claims of the ubiquitous presence of microorganisms able to degrade petroleum hydrocarbons in these soils.

In the studies contained in this thesis, commercially available diesel oil, obtained from a fuel station in Lancaster was used as the complex contaminant mixture. Diesel oil consists of a broad range of POPs, of which PAHs represent an important class, many which are toxic and/or carcinogenic, and may be resistant to biological, chemical and photolytic reactions (Semple *et al.*, 2003; Mahanty *et al.*, 2011). The persistence of

PAHs in the soil environments is largely due to the low aqueous solubility and high sorptive capacity, as well as the chemical recalcitrance, which increases as the number of fused rings increases (Wilson and Jones, 1993). In addition to the physicochemical properties which affect PAH bioaccessibility, other factors including environmental and edaphic conditions (soil structure, pH, temperature, moisture content, inorganic nutrients), co-contaminant type and concentration, and the microbial metabolic versatility influence persistence and/or overall rate of removal of PAHs in soils (Bamforth and Singleton, 2005).

The effects of soil–contaminant contact time and contaminant concentration on biodegradation of PAHs have been widely studied. Most of these studies, however, have been conducted using binary or tertiary mixtures of hydrocarbons, with only a few conducted in soils containing complex mixtures of hydrocarbons, which reflect environmental scenarios and are more representative of soils polluted with diesel, coal tar or creosote. Collectively, results from Papers II, III and V show that the development of indigenous catabolic activity for target hydrocarbons is dependent on the initial diesel concentration and soil contact time, as well as on the hydrocarbon chemical structure. Consistent with some other studies, Paper IV shows that the overall metabolic response of soil microorganisms is influenced by the initial diesel concentration and soil–contact time.

Most studies on the cyclodextrin-enhanced bioaccessibility and biodegradation of PAHs have been conducted with LMW-PAHs and the findings that bioavailability/bioaccessibility is the major factor governing biodegradation is often generalised; however, studies reported in this thesis indicate this may not be true for all PAHs. Papers V, VI, VII and VIII demonstrated the benzo[a]pyrene degradation is dependent, to a greater extent, on the presence of a catabolically-versatile microbial

consortium and substrates that can sustain their growth and/or induce certain enzymatic reaction in them, and less on the bioaccessibility of the contaminant.

In this thesis, it is reported that prescribed fire (200–250 °C; 30 min) has minimal long-term effects on the development of PAH degradative ability of indigenous soil microorganisms in diesel oil-contaminated soil (Paper IX). This study is novel in that, for the first time an investigation of the impact of prescribed fire is focussed on the capacity of soil to degrade the residual oil left in burnt soils. Paper X attempts to make a bold contribution to the on-going discourse on the propriety of applying a fixed k_{EC} value to estimate biomass-C size in soil and sediments. In contrast to the submission of Joergensen *et al.* (2011) that the commonly used fixed k_{EC} value (e.g., 0.45) is suited to all soil types and conditions, the finding of this study advised the use of *in-situ* derived k_{EC} values, in that, the concentration of contaminants in soil (in this case diesel) can significantly influenced the chloroform fumigation-extraction process, leading to overestimation of biomass-C.

5. Recommendations for future research

Based on the findings reported in this thesis, the following recommendations are given for further studies:

i. In Papers II and V–IX, the background soils showed no measurable mineralisation of ^{14}C-benzo[a]pyrene: this may not absolutely mean that biodegradation has not occurred. In future studies, reverse-phase high performance liquid chromatography (RP-HPLC) analysis should be used to reveal the transformations (if any) in the compound; such investigations can also confirm whether ^{14}C-benzo[a]pyrene mineralisation proceeds via cometabolic degradation and what metabolites are formed.

ii. In Papers II–IV, the highest diesel concentration investigated (10,000 mg kg^{-1}) had significant short-term negative effects on the overall metabolic activity as well as on the hydrocarbon degradative ability of the indigenous soil microorganisms. More robust investigations on different soil types and over wider ranges of diesel concentrations should be carried out to establish the long-term effects (i.e., in years).

iii. In Papers II and III, a new parameter, the C_{effect}, was introduced, which provided a better illustration and explanation of the effects of diesel concentration on the initiation and expression of hydrocarbon catabolic activity than the conventional indices of mineralisation (e.g. the lag phase, maximum rate and extent). The C_{effect} is defined as the ratio of mineralisation rates in a polluted soil to the rates in the unpolluted soil; an increase (>>>1) or a decrease (<<<1) in the C_{effect} indicates enhancing or repressing effect, respectively. Analysing mineralisation data using the C_{effect} parameter will give additional and practical insights to the spatial and/or temporal localisation of indigenous catabolic activity in soil with varying contaminant concentrations.

iv. In Paper V, effort to isolate benzo[a]pyrene-degrading bacteria using the traditional culture-dependent techniques was unsuccessful, particularly for the Nether-Kellet soil in which the measured extent of ^{14}C-benzo[a]pyrene mineralisation was comparatively higher than previously reported in other background soils. Culture-independent and molecular techniques to isolate and characterise potential benzo[a]pyrene-degrading bacteria will be of great value.

v. In Papers VI and VII, the presence and increasing concentrations of HP-β-CD did not only fail to promote benzo[a]pyrene mineralisation, it actually negatively affected benzo[a]pyrene biodegradation in three of the four soils investigated. This has weighty ecotoxicological implications for cyclodextrin-aided bioremediation of PAH-contaminated soils. Because of the limited knowledge about the nature and stability of HP-β-CD–benzo[a]pyrene inclusion complexes and how environmental and edaphic factors affect these, as well as the effect of co-contaminants, there is need for further investigation of the variables influencing the physical, chemical and biological interactions of cyclodextrin–PAH–soil under complex contaminant mixture systems. Future research should also include molecular studies of the effects on the microbial community, and toxicological studies of the effects on sentinel soil-dwelling and aquatic organisms; field-polluted samples should be studied.

vi. Based on Papers IV and X, the use of *in-situ* k_{EC} values is advised when quantifying soil microbial biomass-C in soils contaminated with diesel oil. Further studies using δ^{13}C-carbon and ^{14}C-carbon aged oil-contaminated soils and the procedures that can concurrently and differentially quantify C from the various sources may provide a more detailed understanding of the influence of organic contaminants concentration on the quantification of soil microbial biomass-C by chloroform fumigation-extraction (FE) technique. It will also be valuable to evaluate the influence of various organic and metal contaminants with the prospect of obtaining a series of k_{EC} values for a better quantification and comparative analysis of microbial biomass-C in soils, depending on the dominant contaminant(s).

vii. In Paper IX, low-severity prescribed fire was shown to have negligible long-term impact on PAH degradative ability of indigenous soil microflora despite significantly reducing the PAH burden in a diesel oil-amended soil. There are a number of research questions to explore in order to gain further understanding of the prospects and limitations of prescribed fire as an oil-contaminated land remediation operation, as being done for this practice in natural resource management studies.

viii. There are yet so many unknowns about the genetic and regulatory mechanisms involved in biodegradation/cometabolism of benzo[a]pyrene, especially in complex mixtures of co-contaminants; investigations using culture-independent and molecular techniques (e.g., stable isotope probing, nuclear magnetic resonance and mass spectral analyses) are required to determine the role and full degradative potential of un-culturable microorganisms and the mechanisms by which they metabolise HMW-PAHs; examination of the role of extra-chromosomal genes (e.g. plasmids) in B[a]P degradation is also of importance.

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Paper I

Enhancing benzo[a]pyrene bioaccessibility and biodegradation: applicability and limitations of solubility enhancement agents in bioremediation of soil and sediment

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12 **Abstract**

13 Benzo[a]pyrene (B[a]P), a five-ring polycyclic aromatic hydrocarbon (PAH), is of a
14 particular ecological and ecotoxicological interest; due to its bioaccumulation potential
15 and the added recalcitrance to biological, chemical and photolytic breakdown, B[a]P
16 constitutes a significant threat to human health as a potent carcinogenic agent. Together
17 with other PAHs, B[a]P is present at high levels in coal tar-, creosote-, and petroleum
18 oil-contaminated environments. A number of investigations have demonstrated that due
19 to its physicochemical properties, such as low water solubility and a high
20 hydrophobicity, B[a]P, like other PAHs, tends to readily adsorb onto or diffuse into
21 soil matrices and, as a consequence, is scarcely available for biodegradation. Other
22 studies, however, have reported that soil-dwelling and soil-feeding invertebrates, as
23 well as higher animals and plants can still accumulate these microbially-inaccessible
24 pollutants in their tissues. The application of solubility enhancement agents (SEAs) like
25 surfactants and cyclodextrins has been suggested as a promising technology; albeit,
26 variable and conflicting laboratory results alongside the complicated influences of
27 unpredictable edaphic and environmental factors, may limit field deployment to PAH-
28 contaminated soils and sediments. Further, there are only a handful of studies in the
29 literature on SEA-assisted biodegradation of high molecular weight PAHs, making
30 feasibility assessment of SEA-assisted bioremediation a challenge. Therefore, drawing
31 from the relevant literature, the focus of this paper is directed at the applicability and
32 limitations of SEAs in B[a]P biodegradation in soils and sediments.

33

34 **Keywords**: *Benzo[a]pyrene (B[a]P)*; *Bioaccessibility*; *Bioavailability*;
35 *Biodegradation*; *Cyclodextrin*; *Polycyclic aromatic hydrocarbon (PAH)*; *Sediment*;
36 *Soil*; *Solubility enhancement agent (SEA)*; *Surfactant*

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) consist of two or more fused benzene rings and/or pentacyclic molecules that are arranged in linear, angulate or clustered arrays (Figure 1) [e.g., 1,2]. Benzo[a]pyrene (B[a]P), a five-ring PAH, is of a particular ecological and ecotoxicological interest; due to its bioaccumulation potential and the added recalcitrance to biological, chemical and photolytic breakdown, B[a]P constitutes a significant threat to human health as a potent carcinogenic agent [3]. Together with other PAHs, B[a]P is present at high levels in coal tar-, creosote-, petroleum oil-contaminated environments [1,3]. A number of investigations have demonstrated that due to its physicochemical properties, such as low water solubility and a high hydrophobicity (see Table 1), B[a]P, like other PAHs, tends to readily adsorb onto or diffuse into soil matrices and, as a consequence, is scarcely available for biodegradation [3-8]. Other studies however, reported that soil-dwelling and soil-feeding invertebrates, as well as higher animals and plants can still accumulate these microbially-inaccessible pollutants in their tissues [9-19]. Therefore, there is need to ensure thorough clean-up of contaminated soils and sediments.

Central to effective bioremediation of PAH-contaminated soils and sediments are two major issues: (i) inherent microbial biodegradability and, (ii) contaminant bioavailability/bioaccessibility. There are several comprehensive reviews of the factors influencing PAHs (including B[a]P) biodegradation, highlighting means of overcoming or improving the inherent microbial degradative potential of soils and sediments [3-8]; hence, this paper will only attempt to summarily review the key factors as they relate to B[a]P biodegradation. A number of studies have reported encouraging effects of soil flushing with solubility enhancement agents (SEAs) on PAH-contaminated soils *in situ* and *ex situ*; however, far less research attention has been paid to the applications of

SEAs to assist in bioremediation of high molecular weight (HMW)-PAHs. Hence, a comprehensive review of studies on the application of SEAs to enhance PAH bioaccessibility and biodegradation in soils and sediments is presented with particular attention directed at the HMW-PAH, B[a]P.

This review is structured (i) to briefly consider the factors influencing PAH biodegradation in soil and sediments, with particular attention being paid to methods of overcoming the challenges of B[a]P biodegradability and bioaccessibility, and (ii) to discuss the applicability and limitations of SEAs as bioremediation technologies that may be applied to improve the bioaccessibility and the biodegradation of B[a]P in contaminated soils and sediments.

2. PAHs in the environment

PAHs (including B[a]P) have been detected in various environmental media, including air [20-23], water [24,25], sediment [10,25,26], and soil [27-32]. A significantly large portion of PAHs is introduced into the environment through anthropogenic sources including the combustion of fossil fuels (coal and petroleum) and wood (pyrolytic), or through accidental spills of crude or refined petroleum (petrogenic) or discharge from routine storage and transport operations [1,33,34]. In addition, PAHs are ubiquitous at background concentrations in the natural environment, as well as being products of natural or biogenic processes [27,35]. Natural processes, such as forest fires, volcanic eruptions, and during thermal geologic production add to the PAH burdens in soils [4,20,35]. There is also evidence that some PAHs – naphthalene, phenanthrene, and perylene – are produced biologically by termites or associated microorganisms, in certain flowers like the *Magnolia* or different *Annonaceae* species, and in plant debris [36-39].

87 Although, air, water and vegetation may act as environmental sinks for PAHs, soil is
88 the main repository for PAHs [40]. Wilcke [27] hypothesised that the PAH distribution
89 pattern in soil is dominated by two main types, which are indicative of background
90 conditions on the one side (i.e., by biological and diffuse PAHs) and a strong impact by
91 atmospheric deposition of anthropogenic emissions on the other side. The ubiquitous
92 distribution of PAHs in soils is partly due to their being generated from both
93 anthropogenic and natural or biogenic processes in combination with global
94 atmospheric transport phenomena, and partly to their strong hydrophobic affinity and
95 bioaccumulation potential, as well as increased chemical recalcitrance [4,27,41]. Table
96 2 shows the distribution of the priority PAHs (highlighting the percentage of B[a]P) in
97 background soils from across the world.
98
99 *2.1. The special case of B[a]P*
100 From the tonnes of well-controlled laboratory studies in the literature, it is reckoned
101 that virtually all PAHs are biodegradable, co-metabolisable, or at least
102 biotransformable, and that the microorganisms able to transform or degrade PAHs are
103 essentially ubiquitous, and that certain edaphic variables and environmental conditions
104 can affect the rates and/or extents of biodegradation [reviewed e.g. by 3,6,17,42,43-45].
105 Many of these studies have also shown that bioavailability, biodegradability and
106 genotoxicity of PAHs depend largely on their physicochemical properties [e.g.
107 46,47,48]. In general, as aqueous solubility and volatility decreases, and
108 hydrophobicity increases, there is a decrease in bioavailability and biodegradability as
109 number of aromatic rings increases in PAHs. Although, the lower-molecular-weight
110 (LMW)-PAHs (i.e. two or three rings) have acutely toxic effects and may have
111 mutagenic, teratogenic or carcinogenic properties, the active genotoxic/mutagenic

112 members known-to-date are the higher-molecular-weight (HMW)-PAHs (i.e. four to
113 five rings); the five-ring benzo[a]pyrene (B[a]P) being one of the most potent [3,6,49].
114 B[a]P and products of its biotransformation (e.g. the diol epoxides) represent serious
115 risk to human and animal health in that they have high propensity to bio-accumulate in
116 living tissues and exhibit chronic genotoxicity including carcinogenic and immuno-
117 toxic effects [49,50].
118 As compared to the LMW-PAHs, biodegradation of the HMW-PAHs is less extensive
119 in soils and sediments [51,52]. In particular, substantial B[a]P biodegradation is rarely
120 observed in soils, mainly because the pollutant is scarcely available to microbial cells
121 for maintaining their basic metabolic requirements. B[a]P is also thought to be
122 unsuitable as a sole source of carbon and energy to support microbial growth, making
123 degraders to often require additional substrate for growth or induction of enzymatic
124 activity [3]. In addition, some of the intermediates formed during B[a]P
125 biotransformation are more toxic to microorganisms than the parent pollutant, limiting
126 further bioconversion to innocuous materials. Relatively few bacteria, fungi and algae
127 have been reported able to degrade B[a]P; degradation is mostly achieved though
128 cometabolism in the presence of other substrates or in collaboration with other
129 microorganisms [1,3,53,54]. To date, the litter-decomposing basidiomycete *Stropharia*
130 *coronilla* [55] and *Bacillus subtilis* BMT4i [56] are the only organisms reported
131 capable of utilising the compound as sole source of carbon and energy.
132
133 *2.2. Microbial degradation of B[a]P*
134 Microbial degradation is proven to be the principal means of PAH dissipation in the
135 environment, though volatilisation, photo-oxidation, chemical oxidation, sedimentation
136 and bioaccumulation may also play some part [1,4,43]. The ability of indigenous soil

137 microorganisms to degrade most PAHs is believed to be enormous and the ubiquity of
138 PAH-degrading microorganisms has been demonstrated in various contaminated sites
139 ranging from the hot and arid desert Arabian and Kuwaiti soils [57,58], to the tropical
140 and humid Brazilian and Nigerian soils [59,60], to the temperate and wet English and
141 Norwegian soils [61,62], and to the cold and dry Antarctic and Artic soils [63,64].
142 However, in most of the global soils and sediments, pristine or polluted, the inherent
143 ability of indigenous soil microorganisms to degrade B[a]P is either non-existent or
144 very low.
145 Aerobic biodegradation of B[a]P, which require the presence of molecular oxygen has
146 been well documented, mostly in laboratory studies, and the metabolic pathways and
147 enzymatic regulations highlighted [65,66]. Biodegradation of B[a]P within
148 anoxic/anaerobic zones of subsurface soils and sediments is also thought to be possible
149 if the alternative electron acceptors (nitrate, ferrous iron or sulphate) are present and
150 the competent B[a]P-degrading microbial assemblage can survive in the environment
151 [67,68]. Rothermich et al. [67] reported a 24% decline in the initial B[a]P level after
152 338 days incubation of an anoxic, coal tar-contaminated Boston Harbour sediment
153 maintained under sulphate-reducing conditions. A couple of mechanisms for anaerobic
154 degradation of naphthalene have been proposed [69], the mechanism(s) of anaerobic
155 biodegradation of B[a]P remain unexplained. The involvement of plasmids in the
156 degradation of PAHs with molecular weights up to 202 mg mol^{-1} has been reported;
157 however, little is known about the role of plasmids in microbial catabolism of B[a]P
158 [56].
159 A number of studies have demonstrated varying extents of aerobic cometabolic
160 degradation and/or mineralisation of B[a]P by pure and mixed cultures of bacterial
161 genera including *Agrobacterium*, *Bacillus*, *Beijernickia*, *Burkholderia*,

Flavobacterium, *Mycobacterium*, *Pseudomonas*, *Rhodococcus*, *Sphingomonas*, and *Stenotrophomonas*, mostly in liquid media containing growth substrates like phenanthrene, succinate biphenyl, pyrene, and fluoranthene yeast extract [e.g. reviewed by 3]. The algal species *Selenastrum capriconutum* was shown to biotransform B[a]P in the presence of glucose yeast extract [70]. Fungi are also known to play a significant role in B[a]P biodegradation in soils and sediments [as reviewed by 71]. A number of fungi including members of the genera *Aspergillus*, *Candida*, *Cunninghamella*, *Neurospora*, *Nematoloma*, *Penicillium*, *Phanerochaete*, *Pleurotus*, *Saccharomyces*, *Syncephalastrum* and *Trametes* have been reported to degrade B[a]P in the presence of other growth substrates [3].

In general, aerobic bacterial degradation of B[a]P proceeds by the initiation of oxidation with aromatic ring-hydroxylating dioxygenases (ARHDs) to form *cis*-dihydrodiols, via dehydrogenation to form dihydroxylated intermediates, and then via catechol (*ortho* and *meta*-pathways) to produce carbon dioxide and water [4]. In a few cases, for example in *Mycobacterium* sp., B[a]P degradation can also follow the initial oxidation with cytochrome P_{450} monooxygenases to form the *trans*-dihydrodiols. Fungal degradation of B[a]P essentially are of two types. (i) Non-ligninolytic fungal degradation proceeds via cytochrome P_{450} monooxygenases catalysis to produce arene oxide, similar to the mammalian metabolism of PAHs. This is commonly associated with detoxification and later excretion of the metabolites formed, and therefore, it does not usually entail the mineralisation of the initial substrate. (ii) Ligninolytic fungal degradation proceeds by means of lignin and manganese peroxidases and laccases excreted by white-rot fungi to form quinones rather than dihydrodiols, a process that may also lead to mineralisation. Whereas complete mineralisation, albeit minimal, of B[a]P by a single microbial species is rare [24,55], cometabolic mineralisation of B[a]P

187 in the presence of diesel oil by pure or mixed culture microbial consortium [72,73], and
188 recently, by indigenous microorganisms (Papers II, V–IX) has been demonstrated in
189 soils.
190
191 **3. Factors affecting bioremediation of PAH-contaminated soil and sediment**
192 Bioremediation, i.e. the process (including techniques and technologies) of enhancing
193 microbial degradation of contaminants for the clean-up of contaminated land, has been
194 demonstrated at both laboratory- and field-scale to possess several advantages over
195 physical-, chemical- or plant-based approaches [1,5]. Bioremediation of PAH-
196 contaminated soils and sediments can be accompanied in a variety of ways, either *in*
197 *situ* or *ex situ*, such as land farming, composting, prepared-bed bioreactor and slurry-
198 phase bioreactor [43,74,75]. It has since been proven that bioremediation is a
199 remarkably good remediation approach in over 135 Superfund and Underground
200 Storage Tank (UST) sites, as well as many other sites contaminated with complex
201 mixtures of PAHs [76-78]. Nevertheless, the effectiveness of bioremediation
202 technology to treat contaminated sites is plagued with a number of challenges [79-81].
203 In the soil and sediment environments, PAHs may be loss through biodegradation,
204 leaching, photo-oxidation or volatilisation, or it may accumulate within soil-dwelling
205 biota or be retained or sequestered in soil's mineral and organic matter fractions [1,82].
206 In addition to the physicochemical properties (e.g. aqueous solubility, polarity,
207 hydrophobicity, lipophilicity and molecular structure) of the PAHs, the individual
208 contribution of these loss processes depends on other factors broadly grouped into
209 (micro)biological, edaphic and environmental. Biological or microbiological factors
210 include the abundance, structure and functional diversity of the microbial community,
211 the physiological status and degradative capability of the various populations within

212 the microbial community as well as the abundance of microbial predators like
213 protozoans, and the type and abundance of other soil-dwelling organisms like
214 earthworms. Edaphic factors include, soil type (texture, mineral and organic matter
215 content) and depth, pH, moisture, oxygen and inorganic nutrients (nitrogen,
216 phosphorus, potassium) contents, and effective cation-exchange capacity (ECEC), as
217 well as the presence, composition and concentration of co-contaminants, such as
218 hydrophobic organic compounds (HOCs) and metals, and the quality and quantity of
219 other carbonaceous geosorbents like black carbons (BCs) and kerogen. In general,
220 environmental factors include climatic variables like, ambient temperature, wind
221 conditions, sunlight and precipitation intensity, as well as vegetation type and density,
222 topography and other hydrogeological variables [8,83,84].
223 The effects, individually or combined, of these factors on B[a]P biodegradation have
224 been studied to varying extents at the laboratory- and field-levels, and have been
225 reviewed comprehensively elsewhere [3-7]. In field contaminated sites, the extent to
226 which B[a]P is available for interactions with microorganisms, and the degree to which
227 it is degradable are dependent upon the PAH's physical and chemical properties in
228 addition to the specific degradative properties of the extant microbial community as
229 well as other site-specific edaphic factors and environmental conditions [85].
230 Furthermore, the presence of co-contaminants, such as other HOCs and metals, natural
231 sorptive materials like BCs, natural mobilising agents like dissolved organic matter
232 (DOM), or other more soluble and readily-degradable carbon substrates, can influence
233 both the bioavailability and biodegradation of B[a]P by acting as competitive inhibitors
234 or preferential substrates, or as co-metabolites [86], or providing further sequestering
235 phases [87-91], or aiding further contaminant mobility [92], or promoting faster
236 microbial growth [93,94], respectively. Edaphic and environmental factors may affect

237 the rate of biodegradation directly or indirectly by influencing the behaviour and the
238 availability of PAHs and/or the survival, abundance and the activity of the degrading
239 populations [95-97]. Photosensitisation can increase toxicity of B[a]P to human
240 epithelial cell and natural microbial assemblage, but can also increase B[a]P
241 mineralisation rate in a river water containing riboflavin twice as that without
242 riboflavin [102]. Recently, Lily et al. [56] demonstrated that UV-mediated photolysis
243 enhanced mineralisation of B[a]P in *Bacillus subtilis* BMT4i by 1.5-fold when grown
244 in basal salt medium broth for 7 days.
245 Approaches to improve the metabolic barrier to the biodegradation of PAHs (including
246 B[a]P) in contaminated soils and sediments can be broadly categorised as
247 bioaugmentation and biostimulation, and are reviewed in detail elsewhere [42,43,98,
248 99]. Bioaugmentation, i.e., the introduction of organisms with greater or enhanced
249 catabolic activity, and biostimulation, i.e., the supplementation with requisite nutrients,
250 electron acceptors and/or readily-degradable carbon substrates to promote increased
251 catabolic activity or support faster proliferation of the indigenous microbial
252 community, have been used to improve microbial degradation of B[a]P in soils and
253 sediments [100,101]. Microbial adaptation aided by previous exposure to analogous
254 chemicals and/or genetic modification is essential for rapid and extensive degradation
255 of B[a]P in soils and sediments. In addition, presence of suitable co-metabolites, at the
256 right concentrations, is crucial for the initiation and progression of B[a]P
257 biodegradation, since this compound on its own cannot readily support microbial
258 proliferation [3,42,53].
259

4. Overcoming limitations of bioavailability/bioaccessibility to B[a]P biodegradation in soil and sediment

The concepts of bioavailability/bioaccessibility and the implications for risk assessment and bioremediation of contaminated land have been extensively reviewed by other authors [82,103,104]. There are several reviews [e.g., 33,105,106], which have dealt with ways of overcoming PAH bioavailability, focussing more on the LMW-PAHs and cited studies mostly carried out in water, with few examples in soil slurry. Little attention, however, has been paid to means of improving bioaccessibility of B[a]P in bioremediation of soils and sediments.

Due to the physicochemical properties, such as low water solubility (3.8 μg l^{-1}) and high hydrophobicity (log K_{ow} 6.06), B[a]P, like other PAHs, tends to readily sorb to soil organic carbon (SOC) or diffuse into soil micropores by numerous physical and chemical interactions [107,108]. The use of SEAs, including, surfactants, cyclodextrins and co-solvents, to increase PAH solubility is one option to improve remediation of soils contaminated with PAHs [105,109,110]. Studies showing evidence of successful applications of SEAs as a stand-alone remediation technology to increase the apparent solubilisation and removal from soils of HOCs including PAHs have recently been reviewed [74]; therefore, this paper focuses mainly on the application of SEAs to enhance PAH bioaccessibility for microbial remediation. As there is limited research on SEA-assisted biodegradation of HMW-PAHs, especially B[a]P, the applicability and limitations of this remediation approach is discussed with respect to the relevant literature on LMW-PAHs, where appropriate.

283 **4.1. Surfactant-assisted techniques**

284 The bioaccessibility of PAHs including B[a]P in contaminated soils can be enhanced
285 by adding certain organic compounds to facilitate increased solubilisation of the
286 pollutants; some of these compounds are surface active agents (i.e. surfactants), which
287 incorporate the pollutants into the hydrophobic core of micelles [3,110]. Surfactants are
288 usually organic compounds that are amphiphilic – consisting of both hydrophobic
289 groups (i.e. "tails") and hydrophilic groups (i.e. "heads") – making them soluble in
290 both organic solvents and water. Surfactants reduce the surface tension of water by
291 adsorbing at the liquid–gas interface, or reduce the interfacial tension between oil and
292 water by adsorbing at the liquid–liquid interface. Typical desirable surfactant
293 characteristics include low surface tension, low critical micellar concentration (CMC),
294 as well as low adsorption to soil or sediment and low soil dispersion [110]. In addition,
295 ideal surfactants should be of low cost and toxicity, and relatively non-biodegradable to
296 assist in recovery and re-use for the process to be economical. Some of the
297 physicochemical properties of common surfactants are presented in Table 3. The
298 mechanisms of interaction between surfactant molecules and HOC molecules could be
299 via micellar solubilisation and/or emulsification; the relative importance of both
300 mechanisms is difficult to appreciate but surface solubilisation could dominate when
301 PAHs are involved [111]. Above the CMC, surfactant molecules form aggregates or
302 micelles; the presence of micelle increases the apparent solubility, facilitating the
303 uptake of the hydrophobic solutes into the microbial cell [111].

304 Surfactant-assisted bioremediation has been suggested as a promising technology for
305 the clean-up of contaminated soils and sediments [110,112,113]. Table 4 presents the
306 five groups of commonly used surfactants including non-ionic, anionic, cationic, bio-
307 surfactant, and mixed-type surfactants and their performance on PAH biodegradation

308 [105]. The hydrophilic portions of anionic surfactants contain sulphate, sulfonate or
309 carboxylate; cationic surfactants contain quaternary ammonium; and nonionic
310 surfactants contain polyoxyethylene, sucrose, or polypeptide. Generally, the
311 hydrophobic parts of surfactants contain paraffins, olefins, alkylbenzenes,
312 alkylphenols, or alcohols. Commercially available anionic surfactants include Dowfax
313 8390, sodium dodecyl benzene sulfonate (SDBS), sodium dodecyl sulphate (SDS),
314 linear alkylbenzene sulfonates (LAS), monoalkylated disulfonated diphenyl oxide
315 (MADS-C12), dialkylated disulfonated diphenyl oxide (DADS-C12) and Steol 30;
316 cationic surfactants include benzyldimethyldodecylammonium bromide (BDDA),
317 myristylpyridinium bromide (MPB), dodecyltrimethylammonium bromide (DTAB),
318 tetradecyltrimethylammonium bromide (TDTMA), hexadecyltrimethylammonium
319 bromide (HTAB) and octylamine; and nonionic surfactants include Triton X-100, the
320 Tween series (40, 80) the Brij series (30, 35), Arkopa N-300, Sapogenat T-300,
321 Tergitol 15S7, Tergitol NP-10, T-Maz 20, CA 620 and TerraSurf 80.
322
323 *4.1.1. Anionic and cationic surfactants*
324 Anionic and cationic surfactants are very effective at removing metals and organic
325 contaminants during soil washing/flushing [114]; however, most studies of SEA-
326 assisted biodegradation indicate that the applicability of both anionic and cationic
327 surfactants is limited, in that, they are particularly toxic to microorganisms and other
328 ecological biota, and that cationic surfactants are likely to be adsorbed by soil particles
329 due to their charges while anionic surfactants may precipitate in soil or in hard
330 subsurface water [110,114,115]. The anionic surfactant, LAS was reported to have
331 short-term negative effects on soil microbiology in sewage sludge-amended
332 agricultural soil [116,117]. The efficiency of ionic surfactants is thought to relate to the

size of their hydrophobic core and to the accessibility of the inner core. For a given alkyl chain length, monosulfonates exhibit a lesser solubilisation of PAH than alkyldiphenyl oxide disulfonates [111].

The chemical structure, the shape and size of micelles, and the CMC values of anionic surfactants affect their solubilisation capacity. Lan Chun et al. [118] investigated the solubilisation capacity of three structurally-different anionic surfactants for single, binary and ternary PAH mixtures. The anionic surfactants studied include the conventional surfactant SDBS with one hydrophilic head group and one hydrophobic tail; the gemini surfactant MADS-C12 with two hydrophilic head groups and two hydrophobic chains; and the dianionic surfactant DADS-C12 with two hydrophilic head groups and one hydrophobic tail. The authors reported that the apparent aqueous solubilisation of naphthalene or phenanthrene was enhanced in the order of SDDS < MADS-C12 < DADS-C12, suggesting that the hydrophobic chains in micellar core play more important role for the solubilisation of PAHs than the benzene rings in the palisade layer of a micelle. It was also observed that for the binary and ternary PAH mixtures (e.g., naphthalene, phenanthrene and/or pyrene), in the presence of less hydrophobic solutes, the micellar partitioning of the more hydrophobic solute was increased [118]. The remarkable difference in the solubilisation capacity and CMC values of the surfactants for single and multiple PAH systems suggests that the assessment of a surfactant's potential for remediation should be based on its capacity in the presence of multiple contaminants [118].

Studies have shown varying results (ranging from enhancements, to no effects, and to inhibitions) of ionic surfactant-assisted biodegradation of PAHs, indicating the dependence on surfactant–bacterial species combination, surfactant concentration, pollutant type, soil type and conditions. During 32 day incubation at 10 °C, low SDS

358 concentrations (50–100 mg l^{-1}) significantly enhanced diesel oil biodegradation,
359 whereas higher concentrations (500–1000 mg l^{-1}) inhibited hydrocarbon biodegradation
360 by a psychrotrophic inoculum in liquid culture [119]. Oil biodegradation by the
361 indigenous microorganisms in an Alpine soil was inhibited at all SDS concentrations
362 investigated; meanwhile, the surfactant itself was rapidly biodegraded during the first
363 5–15 days both in the liquid culture and in the soil [119]. Higher SDS concentrations
364 promoted greater release of hydrocarbons from soil colloids. Remarkably, increased oil
365 biodegradation at the low SDS concentrations in liquid medium occurred only after 10
366 days, well after the SDS had been fully degraded within the first 4–7 days. This
367 indicates either the effect of reduced toxicity at low SDS concentrations or increased
368 growth of the organisms on low SDS concentrations. The inhibition observed at higher
369 SDS concentrations in liquid medium and at all SDS concentration in soil was
370 attributed to both increased toxicity and the accumulation of inhibiting metabolites
371 during the course of SDS biodegradation [119].
372 In a pollutant mobilisation study, SDS solutions (0.005 to 1% w/v) increased the
373 desorption of PAHs up to four-rings, and increase in SDS concentration resulted in the
374 mobilisation of very low water-soluble five- and six-ring PAHs in a wood-preserving
375 soil contaminated with creosote for a period of at least 20 years [120]. However, in the
376 corresponding biodegradation test, SDS (100 and 500 mg kg^{-1}) significantly decreased
377 the biodegradation of fluorene, phenanthrene and all of the four-ring PAHs [120,121].
378 PAHs with more than four rings were not biodegraded in the presence or absence of
379 SDS, indicating that though SDS may be efficient in mobilising HMW-PAHs in aged
380 contaminated soils, it may not be effective to enhance HMW-PAH biodegradation. In
381 another study, SDS significantly increased solubilisation but inhibited biodegradation
382 of phenanthrene because SDS was preferred as a growth substrate by the adapted

383 mixed bacterial culture [122]. The inhibition also increased with SDS concentration,
384 and resulted in acidification of the medium with prolonged incubation, indicating that
385 the products of SDS hydrolysis can also be inhibitory to microorganisms [122].
386 The anionic surfactant LAS had no beneficial effect on phenanthrene degradation in
387 liquid medium due to its preferential utilisation by *Mycobacterium* sp. KR2 at low
388 concentrations ($\leq$10 mg l^{-1}) and to its increased toxicity at high concentrations (20–900
389 mg l^{-1}) [123]. Growth of *Mycobacterium* sp. KR2 was not affected at LAS
390 concentrations of 0–40 mg l^{-1} but was significantly delayed or inhibited by LAS at
391 higher levels ($\geq$80 mg l^{-1}) [123].
392 Recently, Pelaez et al. [101] conducted a pilot-scale study to evaluate the effects of two
393 biostimulants, a slow- and a fast-release fertiliser (SRF and FRF, respectively)
394 combined with two commercial surfactants, Ivey Sol and Bioversal on PAH
395 biodegradation. After 60 days of incubation, $\sum$PAHs removal increased from 56.2% in
396 SRF-only amended soil to 72.6% and 78.3% in SRF + Ivey Sol and SRF + Bioversal
397 amended soils, respectively. Similarly, naphthalene removal increased from 60.6% in
398 SRF-only amended soil to 80.1% and 87.0% in SRF + Ivey Soil and SRF + Bioversal
399 amended soils, respectively. However, the effect on five–six-ring PAHs was not as
400 significant; the increased was from 21.7% in SRF-only amended soil to 26.8% and
401 26.8% in SRF + Ivey Soil and SRF + Bioversal amended soils, respectively [101].
402 Following the scale-up of the study to an "on-site" bioremediation program using
403 biopiling and amendment with FRF and Ivey Sol, there was significant biodegradation
404 of $\sum$PAHs: 98% naphthalene, 84% three-ring PAHs, 74% four-ring PAHs and 74%
405 five–six-ring PAHs, with respect to the initial contaminants levels in soils was
406 achieved after 161 day treatment [101].

407 The cationic surfactant, TDTMA exhibited greater toxicity to *Mycobacterium* sp. KR2
408 than the anionic surfactant LAS and nonionic surfactants Tween 80, Brij 30, Brij 35
409 and 10LE [123]. The authors reported that the inhibition of phenanthrene degradation
410 by TDTMA at concentrations ($\leq$40 mg l^{-1}) well below its CMC (100 mg l^{-1}) was not
411 due to surfactant toxicity per se, but as a result of the preferential utilisation of the
412 surfactant at low levels as non-toxic nutrient resource. When sorbed at low levels to
413 natural solids (e.g., soils and bentonite), the cationic surfactant MPB, behaved as a
414 more powerful medium for sorbing organic contaminants (phenol, *p*-nitrophenol and
415 naphthalene) than did the dissolved surfactant in micellar form. At the low levels on
416 solids, especially bentonite clays, MPB functions effectively as an adsorptive surface
417 rather than a partition phase. The reverse becomes true for the sorbed surfactant at high
418 loading in solids [124].
419 In a study to compare the solubilisation capacity of three surfactants, the cationic
420 DBBA, the anionic SDS and the nonionic Triton X-100 for B[a]P, DBBA was found to
421 be the most efficient towards solubilisation in micellar phase [111]. The authors
422 attributed this to the contribution of two mechanisms: a partition process into the
423 hydrophobic core of the micelle and a surface solubilisation into the palisade layer due
424 to specific interactions, surface solubilisation being the major contribution. Meanwhile,
425 at relatively low concentrations, anionic surfactant SDS (0.2 g l^{-1}) and cationic
426 surfactant CTAB (0.01–0.05 g l^{-1}) exhibited high toxicity to a *Stenotrophomonas*
427 *maltophilia* able to degrade HMW-PAHs [125].
428
429 *4.1.2. Nonionic surfactants*
430 For field application, nonionic surfactants are often preferred, and the most frequently
431 used in PAH biodegradation as they are less toxic to microorganisms and less

biodegradable than other types of surfactants [123,125,126]. In addition, nonionic surfactants usually have greater solubilisation capacity than ionic surfactants; strongly hydrated shell of ionic surfactants reduces the accessibility of the core and thus the solubilisation when compared to nonionic ones, but nonionic surfactants are more likely to adsorb onto clay fractions compared to anionic ones [126]. Sorption of surfactants to soil may result in a proportion being unavailable for micellar solubilisation of PAHs [127], hence reducing the efficiency in solubilisation.

The results from research available in the literature indicate that the effectiveness of the application of nonionic surfactants to bioremediation of PAH-contaminated soils is often variable and conflicting. Addition of nonionic surfactants above their respective CMCs has been demonstrated to inhibit, enhance, or have no tangible effect on PAH biodegradation in soils and sediments, depending on the surfactant type and concentration, PAH type and concentration, soil type and conditions, moisture content, and the type of microorganisms present.

Allen et al. [128] observed the contrasting effects of Triton X-100 during biodegradation of the PAHs naphthalene and phenanthrene, by two dioxygenase-expressing bacteria, *Pseudomonas* sp. strain 9816/11 and *Sphingomonas yanoikuyae* B8/36. Triton X-100 increased the rate of oxidation of the PAHs by strain 9816/11 with the effect being most noticeable when phenanthrene was used as a substrate, but inhibited the biotransformation of both PAHs by strain B8/36 under the same conditions. Triton X-100 also inhibited growth of the wild-type strain *S. yanoikuyae* B1 on aromatic compounds. Further studies indicate that Triton X-100 increased dioxygenase activity in strain 9816/11 but did not inhibit dioxygenase enzyme activity in strain B8/36 *in vitro*. This led the authors to suggest that the difference in the structure of the cell wall in these microorganisms was responsible for the contrasting

457 effects [128]. Toxicity of many surfactants is mainly due to membrane-damaging
458 effects [129].
459 In a study to compare the ability of a number of nonionic surfactants to solubilise and
460 enhance microbial degradation of PAHs in liquid medium, Tiehm [122] found that
461 while all nonionic surfactants tested solubilised the PAHs, the rate and extent of PAH
462 biodegradation were affected by the toxicity of surfactants, with toxicity increasing as
463 hydrophobicity decreases, i.e., with increasing ethoxylate chain length. All surfactants
464 investigated enhanced the degradation of phenanthrene or fluoranthene by their
465 respective mixed culture, but biodegradation of fluorene or pyrene was depended on
466 the surfactant used, indicating that nonionic surfactants may exhibit some toxicity to
467 specific PAH degraders. The nonionic surfactants Malipal 013/90 and Triton X-102
468 inhibited growth of *Mycobacterium* sp. and the biodegradation of acetate,
469 phenanthrene, fluoranthene, and pyrene, but Genapol X-150, Brij 35, Arkopal N-300,
470 Sapogenat T-300, Pluronic PE 6400 and Tegopren 5851 enhanced the growth of
471 *Mycobacterium* sp. and the biodegradation of fluorene, phenanthrene, anthracene,
472 fluoranthene, and pyrene [122]. Other researchers have reported selective toxicity of
473 nonionic surfactants to microorganisms in soils and sediments [130,131].
474 Tsomides et al. [131] reported the effect of Triton X-100 at levels above its CMC on
475 the solubilisation and the biodegradation of phenanthrene in sediment. The initial
476 inhibition of biodegradation was followed by a significantly faster mineralisation after
477 5 days in the surfactant-amended as compared to the surfactant-free systems; however,
478 extents of phenanthrene mineralisation were statistically similar in both systems after
479 22 days [131]. The solubilisation capacities of a set of nonionic surfactants increase in
480 the order of Brij 30 < Triton X-100 < Tween 80 < Brij 35, which correlated with their
481 polyoxyethylate chain lengths [132]. All the surfactants significantly increased the

apparent solubilisation of naphthalene, phenanthrene, and pyrene with efficiencies ranging from 21.1 to 60.6%, 33.3 to 62.8%, and 26.8 to 70.9%, respectively. While Brij 35 and Tween 80 inhibited the growth of *Pseudomonas putida,* the bacteria utilised Triton X-100 and Brij 30 as sole carbon and energy sources at concentrations above their CMCs [132]. Whereas *Pseudomonas putida* demonstrated competence to mineralise pyrene (28%) in a liquid medium without surfactants, Triton X-100, Tween 80 and Brij 35 inhibited pyrene mineralisation by the bacteria, and the fraction of the micellar-phase PAH that can be directly biodegraded decreases as the concentration of micelles increases. Meanwhile, pyrene mineralisation was enhanced with Brij 30 in soil [132].

In a study to evaluate the effect of nonionic surfactants on solubility and biodegradation of PAHs in aqueous and soil slurry, naphthalene and phenanthrene solubility increased linearly with increasing surfactant concentrations in the range of 0.25–2 g l^{-1} [133]. In similar manner, surfactant solubility increased as the hydrophile–lipophile balance (HLB) values decreased, indicating the effect of surfactant type and concentration [133]. Brij 30 showed the greatest desorption capacity for phenanthrene, followed by Tween 80 and then Triton X-100, correlating to their HLB values. Biodegradability of the nonionic surfactants also followed the trend observed for their desorption capacity. All three nonionic surfactants supported complete biodegradation of naphthalene or phenanthrene by a phenanthrene-acclimatised culture within 60 h in a liquid medium; the capacity to enhance biodegradation increased with increasing surfactant concentrations up to 2 g l^{-1} [133]. In soil slurry systems, Brij 30 promoted faster initial degradation rate of phenanthrene in the sand than in clay soil although the extent of degradation remained comparable after 10 h in both soils [133].

506 In a more recent study, Bueno-Montes et al. [134] evaluated the influence of the
507 nonionic surfactant Brij 35 on biodegradation of slowly desorbing PAHs in
508 contaminated soil from a creosote-polluted site, and a manufactured gas plant (MGP)
509 soil that had previously been treated by bioremediation. At concentration above its
510 CMC, Brij 35 enhanced the biodegradation of slowly desorbing PAHs in suspensions
511 of both soils, being especially efficient with the bioremediated soil. However, Brij 35
512 inhibited the biodegradation of HMW-PAHs pyrene and B[a]P; this was attributed to
513 the effects of competition with other solubilised PAHs present at relatively higher
514 concentrations [134]. Zhou et al. [135] demonstrated that Triton X-100 significantly
515 enhanced degradation of phenanthrene by *Pseudomonas* sp. ZJF08 because the
516 organisms was not able to degrade the surfactant; the ability of *Pseudomonas* sp.
517 ZJF08 to rapidly degrade SDS and Tween 80 resulted in the surfactant only been able
518 to enhance biodegradation rate at the initial phase.
519 Zhu and Aitken [136] evaluated the ability of two nonionic surfactants; one
520 hydrophobic (Brij 30) and one hydrophilic (C12E8), to enhance the biodegradation of
521 PAHs in a contaminated soil after it had been treated in an aerobic bioreactor.
522 Biodegradation of PAHs with three- and four-rings was significantly enhanced at Brij
523 30 concentrations corresponding to doses below and at its CMC, but biodegradation of
524 the three-ring PAHs only was significantly enhanced at concentration above the CMC.
525 By contrast, C12E8 did not enhance PAH biodegradation at any of the three doses. Brij
526 30 addition, at the lowest dose, significantly increased the desorption of most PAHs,
527 but C12E8, at the lowest dose, actually decreased the desorption of all PAHs [136].
528 The authors highlighted than the properties of the surfactant and its dose relative to the
529 corresponding aqueous-phase concentration are important factors in designing systems
530 for surfactant-assisted bioremediation of PAH-contaminated soils in which PAH

bioavailability is limited [136]. Brij 30 inhibited both microbial growth and HMW-PAHs degradation by indigenous microbiota in a real industrial polluted soil [93]. Tween 80 increased PAHs solubility in dense-slurry system containing a creosote-contaminated field soil and in a soil-free system, but the PAHs were not readily bioavailable to the mixed consortium of microbes indigenous to the creosote-contaminated soil [137].

Like the ionic surfactants, temperature and pollutant type and concentration are shown to affect the effectiveness of nonionic surfactants. At the total concentration of 2 mg l^{-1} of a mixture of two PAHs, the addition of Tergitol NP-10 (100 mg l^{-1}) decreased the extent of mineralisation of anthracene from 48.8 to 41.0% and increased that of pyrene from 66.1 to 71.1% at 25 °C, whereas the extent of anthracene mineralisation was significantly decreased from 18.5 to 11.5% and that of pyrene from 61.5 to 3.8% at 10 °C [138]. At the total concentration of 20 mg l^{-1} of the PAHs, addition of the surfactant significantly increased mineralisation of anthracene from 17.3 to 33.0% and that of pyrene from 7.6 to 27.6% at 25°C, while anthracene mineralisation was slightly increased from 8.7 to 9.6% and pyrene mineralisation significantly decreased from 5.1 to 0.4% at 10 °C [138].

A few studies have reported on nonionic surfactant-assisted solubilisation and biodegradation of HMW-PAHs including B[a]P, with mixed results [56,125,139-141]. At concentrations up to 10 g l^{-1}, the nonionic surfactants Brij 35, Tergitol NP-10, Triton X-100 and Tyloxapol, exhibited little or no effect on growth of *Stenotrophomonas maltophilia* known to degrade HMW-PAHs [125]. Igepal CA-630 caused some inhibition at concentrations of 5 and 10 g l^{-1}; growth inhibition by all surfactants was observed at 20 g l^{-1} [125]. An increment in B[a]P solubilisation of up to 1000 times higher than in water was reported when some nonionic surfactants were

556 added to a MGP soil [142]. While Triton X-100 significantly increased the apparent
557 solubilisation of PAHs ranging from two- to six-rings, it hardly enhanced or, in some
558 cases, even inhibited biodegradation by a microbial consortium of PAHs with
559 molecular weight similar to or higher than benzo[b]fluoranthene [143]. Tween 80
560 increased the mineralisation of the four-ring fluoranthene in shake flask culture by
561 *Pseudomonas alcaligenes* PA-10 from 62.5 to 79.6% [141]. B[a]P degradation by
562 *Phanerochaete chrysosporium* was significantly enhanced in a Tween 80-solubilised
563 system, whereas no tangible degradation occurred in a solid-phase or soil-slurry system
564 without the surfactant [139,140]. Recently, Lily et al. [56] reported that B[a]P
565 degradation by *Bacillus subtilis* BMT4i was enhanced in the presence of 0.01% of
566 either Tween 20 (58.64%) or Triton X-100 (50.12%) as compared to the control
567 (46.32%), but SDS inhibited mineralisation (28.81%).
568 It has to be noted that both PAH–PAH interactions and micelle–PAH interactions
569 influence the concentration of PAH mixtures in micellar surfactant solutions [144].
570 PAH–PAH interactions can influence aqueous solubility, while micelle–PAH
571 interactions can affect the distribution of PAHs in the micellar phase, which may
572 change as the mixture composition changes. Hydrophobicity of PAHs, i.e., their
573 octanol–water partition coefficients, temperature and salinity have also been reported
574 to affect the solubilisation capabilities of nonionic surfactants [145]. The increasing
575 aggregation number and the micellar size at higher temperature result in the higher
576 solubilisation capacity of Tergitol 15S7 [145].
577
578 *4.1.3. Biosurfactants*
579 Naturally, in order to adapt to low availability of substrates, particularly under certain
580 conditions where mass transfer is significantly limited, such as in aged-contaminated

581 soils, many PAH-degrading bacteria may synthesise surface active substances, i.e.,
582 biosurfactants [110,146,147], while some others enhance cell surface hydrophobicity or
583 form biofilms to facilitate growth on the PAHs [148-151]. The role of biosurfactants in
584 microbial survival and metabolism is well-known for a long time, but the application of
585 biosurfactant-aided biodegradation in bioremediation of PAH-contaminated soil is still
586 developing [109]. The growing interest in the use of biosurfactants for environmental
587 applications is mainly because synthetic chemical surfactants are generally considered
588 to be more toxic and require higher concentrations than biosurfactants [105,152];
589 although biosurfactant production cost is about 3 to 10 times higher than that of
590 synthetic surfactants [109,110,153]. Examples of biosurfactants include the glycolipids
591 – rhamnolipids, trehalolipids, sphorolipids; lipopeptides – viscosin, surfactin,
592 subtilisin; polymeric surfactants – Alasan, Emulsan. A number of bacteria and yeast
593 that grow on diesel oil, kerosene, crude oil and oily sludge, and able to produce
594 biosurfactants with the potential for remediation of PAH-contaminated soils have been
595 isolated and characterised [109, 154, 155]. A detail list of biosurfactants and organisms
596 able to produce biosurfactants are available elsewhere [99, 110 and references
597 contained therein].
598 The solubilisation capability of biosurfactants is not in doubt [109]; however, the few
599 studies in the literature on biosurfactant-aided bioremediation of PAHs present mixed
600 results. The effectiveness of biosurfactants to remove PAHs from soils and sediments
601 is dependent on the soil type, molecular structure of the PAH and the concentration of
602 the biosurfactant used [109,110,153]. In general, biosurfactants may be limited to
603 desorbing PAHs with not greater than four-rings and are only effective in assisting to
604 biodegrade LMW-PAHs [109,114].

605 Studies that investigated biosurfactant-aided biodegradation of B[a]P in soil or
606 sediment are rare in the literature [109]. In a recent study, Wong et al. [156] reported
607 that biosurfactants produced by *Acinetobacter calcoaceticus* BU03 significantly
608 improved desorption and solubilisation of phenanthrene and B[a]P to 54.3 mg l^{-1} and
609 2.08 mg l^{-1}, respectively, in water. B[a]P degradation was enhanced, increasing from
610 16.4% in soil without biosurfactant to 83.8% in soil amended with *A. calcoaceticus* or
611 the biosurfactant extract. When the soil was augmented with the biosurfactants and
612 *Bacillus subtilis* B-UM, extent of degradation after 42 days was 65.1% [156]; this
613 indicates that the effectiveness of the biosurfactants is also dependent on the type of
614 organisms present.
615 Rhamnolipids from *Pseudomonas aeruginosa* UG2 in a bioslurry increased the
616 solubilisation of four-ring PAHs more significantly than the three-ring PAHs and were
617 five times more effective than SDS; however, the addition of the biosurfactant did not
618 improve the biodegradation in a wood-preserving soil contaminated with creosote for a
619 period of at least 20 years [121]. This was attributed to the preferential utilisation of the
620 biosurfactant by PAH degraders. Meanwhile, biosurfactant produced by another strain,
621 *Pseudomonas marginalis*, enhanced the solubilisation and biodegradation of
622 phenanthrene [157].
623 The synthetic surfactant Tween 80 failed to increase fluoranthene desorption from soil,
624 whereas the biosurfactant rhamnolipid JBR (0.5 g kg^{-1}) significantly increased
625 fluoranthene desorption (11-fold) and resulted in a greater initial degradation rate of
626 200 mg kg^{-1} of the PAH in soil by *Pseudomonas alcaligenes* PA-10; however extents
627 of biodegradation after 28 days were comparable in soils with or without JBR [141].
628 The addition of phenanthrene (200 mg kg^{-1}) having similar effect on fluoranthene
629 biodegradation led the authors to conclude that the increased solubilisation of the PAH

630 by the biosurfactant and/or utilisation of the biosurfactant as co-metabolite are
631 responsible for the initial increased fluoranthene degradation. The biosurfactant JBR
632 was also reported to have greater enhancing effect on the apparent solubility in liquid
633 medium and mineralisation of fluoranthene by *Pseudomonas alcaligenes* PA-10 than
634 Tween 80 [141]. Although the Pseudomonad grew rapidly on JBR at its CMC of 0.5 g
635 l^{-1}, toxic effect on the degrader was observed at a concentration tenfold higher than the
636 CMC [141].
637 Tween 80 and biosurfactants P-CG3 and P.9027 produced by a *Pseudomonas*
638 *aeruginosa* strain P-CG3 and *Pseudomonas aeruginosa* ATCC 9027, respectively,
639 effectively enhanced the solubility of both phenanthrene and pyrene under thermophilic
640 condition (55 °C), which was linearly proportional to the concentrations of surfactants
641 above their respective CMC [158]. However, in a further investigation, Tween 80 and
642 the two biosurfactants inhibited phenanthrene degradation by an isolate *Bacillus* sp. B-
643 UM; the negative effect increased as surfactant concentrations increased, with
644 complete inhibition of phenanthrene degradation by all the surfactants at concentrations
645 above their respective CMC [159]. While preferential degradation of the surfactants
646 was also reported, the authors postulated that the hydrophobic property of B-UM was
647 responsible for the reduced phenanthrene biodegradation as the surfactants reduced the
648 direct contact between bacterial cells and phenanthrene [159].
649 Rhamnolipids from *Pseudomonas aeruginosa* AT10 enhanced the biodegradation of
650 Casablanca crude oil by a microbial consortium [160]. Biodegradation of total
651 petroleum hydrocarbons after 10 day increased from 32% in the absence of
652 rhamnolipids to 61% when hydrocarbons are emulsified by 500 mg rhamnolipids l^{-1} in
653 liquid medium; the rhamnolipids were particularly effective on the isoprenoid group
654 from aliphatic fraction and the alkylated PAHs from the aromatic fraction. Since the

655 alkylated derivatives are known to exhibit greater acute toxicity and are more
656 mutagenic than the parent PAHs [161], the finding indicates that the potential of
657 biosurfactants may not only be to increase the overall removal of PAHs but to also
658 reduce acute toxicity and mutagenicity of their residuals. The study by Hickey et al.
659 [141] also indicated that fluoranthene toxicity to *Bacillus megaterium* IMD 147 and
660 *Drosophila melanogaster* was reduced in soil treated with JBR and *P. alcaligenes* PA-
661 10 to that in the uncontaminated soil after 28 days
662 A recent study by Portet-Koltalo et al. [114] indicated that while two cyclolipopeptidic
663 biosurfactants, amphisin and viscosin-like mixture, produced from *Pseudomonas*
664 *fluorescens* strains were effective only at desorbing two–three-ring PAHs (naphthalene
665 to phenanthrene), the synthetic surfactant, SDS, additionally desorbed the four-ring
666 PAH, pyrene. In a study using a soil-packed column, soil pH affected the ability of
667 biosurfactants to enhance apparent solubility of PAHs; rhamnolipid solution removed
668 17.3% and 9.5% of phenanthrene from soil with pH 5 and 7, respectively [162]. In a
669 further study to evaluate the effect of residual surfactant after the soil flushing event on
670 microbial processes, phenanthrene degradation by *Sphingomonas* sp. strain 3Y was
671 enhanced except in soil with pH 4 [162]. The structure of rhamnolipids is thought to be
672 strongly dependent on pH, and can undergo changes from large lamellar sheets, to
673 vesicles, and to micelles [110].
674 Gottfried et al. [163] reported that the addition of small amounts of biosurfactant (0.25
675 g l^{-1}) gave a significant increase in phenanthrene removal by *Pseudomonas putida*
676 ATCC 17484 in soil slurries when only biosurfactant was added, but in soil slurries
677 containing salicylate the effects of biosurfactant addition were negligible as there was
678 greater than 90% removal, regardless of the biosurfactant concentration. The finding

679 indicates that the introduction of biosurfactants is unnecessary where carbon substrates
680 to induce metabolic pathway are available.
681
682 *4.1.4. Mixed-type surfactants*
683 At high concentrations, anionic surfactants may precipitate in too hard subsurface
684 water whereas, at low concentrations nonionic could adsorb onto clay materials; a
685 solution to these challenges is the use of mixed surfactant systems [115]. The
686 application of mixed-type surfactants to remediation studies is increasing due to their
687 greater solubilisation and suspension, dispersion and transportation capabilities, as
688 compared to the individual ionic or nonionic surfactants [164,165]. The sorption of
689 nonionic surfactants onto clay materials was observed to decrease with the increasing
690 mole fraction of anionic surfactants in mixed solution, indicating that mixtures of ionic
691 with nonionic surfactants may give a better enhancement [115]. It was observed that
692 the mixture of SDS–Triton X-405 solutions has a larger maximum additive
693 concentration (MAC) and micelle-water partition coefficient (K_{mc}) than predicted by
694 the ideal mixing rule (i.e., obeys Raoult's Law), suggesting a synergistic effect of the
695 micelle on solubilisation of PAHs, which follow the order of pyrene > phenanthrene >
696 acenaphthalene > naphthalene [164]. Although no study has been conducted with
697 B[a]P, it appears that such increasing effect of mixed surfactants as the K_{ow} values of
698 PAH increase could favour B[a]P solubilisation in the presence of other competing
699 PAHs.
700 Other mixed surfactants that have been shown to exhibit synergistic effect on pyrene
701 solubilisation in the micellar-phase are in the order of SDS–Triton X-405 > SDS–Brij
702 35 > SDS–Brij 58 > SDS–Triton X-100, and increases with an increase in the HLB
703 value of nonionic surfactant in mixed systems [165]. More recently, Kabir ud et al.

704 [166] investigated the solubilisation capacities of equimolar mixed micellar solutions
705 of gemini surfactant, $C_{16}H_{33}N^{+}(CH_3)_2$–$(CH_2)_5$–$N^{+}(CH_3)_2$ $C_{16}H_{33}$ $2Br^{-}$ (G), with
706 cetylpyridinium chloride (CPC), bis(2-ethyhexyl)sulfosuccinate (AOT) and Brij 56
707 toward pyrene and anthracene. The equimolar binary surfactant mixtures gave higher
708 solubilisation capacity than their respective individual surfactants, except G–CPC
709 wherein the values were intermediate between the two. Sales et al. [126] also studied
710 the solubilisation of PAHs in water induced by mixed surfactant solutions. The
711 inability of the mixtures between nonionic surfactants Tween 80 or Brij-35 and an
712 amphiphilic modified β-cyclodextrin (Mod-β-CD_{12}) to show synergism in increasing
713 the solubilisation of naphthalene and phenanthrene was attributed to the strong
714 intermolecular interactions in the cyclodextrin aggregates [126]. On the other hand,
715 because these interactions are absent in an anionic fatty acids (sodium laurate), the
716 mixtures formed between it and Tween 80 at all mole fractions investigated produced
717 higher enhancements of naphthalene solubility than the individual surfactants [126].
718 However, Tween 80–fatty acids mixture did not increase phenanthrene solubilisation,
719 indicating the different solubilisation sites of the PAHs in the mixed micelles.
720 Zhao et al. [115] studied the effects of mixed anionic–nonionic surfactants on
721 phenanthrene solubilisation and biodegradation, and reported that the CMC values of
722 all the three mixed surfactants solutions, i.e., SDS–Tween 80, SDS–Brij 35, and SDS–
723 TX100, were sharply lower than that of single SDS solution and exhibited no
724 inhibitory effect on biodegradation of phenanthrene [115]. The inhibition of
725 phenanthrene biodegradation in the presence of 5.0 mM of SDS was attributed to the
726 preferential utilisation of SDS as high CMC and low solubilising capacity would not
727 enhance availability of contaminants significantly [115].

728 Yu et al. [167] demonstrated that the mixture of the anionic surfactant SDS with the
729 nonionic surfactant Triton X-100 did not only improve the solubilisation capacity of
730 the Triton X-100, it also reduced the sorption of Triton X-100 onto soils, resulting in
731 significantly enhanced desorption efficiency of phenanthrene from a contaminated soil.
732 The ability of SDS to improve Triton X-100 solubilisation capacity appears to relate to
733 the mole fraction of SDS in solution. However, the mole ratio of SDS–Triton X-100 is
734 critical to enhance biodegradation of phenanthrene in soil; smaller ratio of 1:9 SDS–
735 Triton X-100 mixed solutions produced significantly higher biodegradation while
736 larger ratio of SDS in the mixed solutions inhibited biodegradation, which may be due
737 to the preferential utilisation of SDS by phenanthrene degraders [167].
738 Naturally-occurring amphiphilic compounds have also been used in combination with
739 synthetic surfactants as mixed-type SEAs to improve HOCs solubilisation and/or
740 enhance their biodegradation. Cho et al. [168] studied the combined effect of natural
741 organic matter (NOM) and surfactants on the apparent solubility of PAHs. The
742 apparent solubilisation of naphthalene, phenanthrene, or pyrene was found to be lower
743 in mixed NOM–anionic surfactant solution than in single NOM solution while the
744 apparent solubility of a PAH in mixed NOM–nonionic surfactant was almost the same
745 as the sum of the PAH solubility in single NOM solution plus its solubility in single
746 nonionic surfactant solution [168]. The authors attributed the decreased solubilisation
747 capacity of mixed NOM–anionic surfactant solution to the fact that cations that are
748 released when the anionic surfactants dissociate may form ion pairs with acidic or
749 phenolic groups in the NOM, increasing the size of these associated-nonpolar moieties,
750 and thus decreasing hydrophobic partitioning of the HOCs into the NOM.
751 Cheng and Wong [169] examined the desorption behaviour of phenanthrene and
752 pyrene in soil–water system in the presence of nonionic surfactant Tween 80 and DOM

753 derived from pig manure or pig manure compost. Addition of 150 mg l^{-1} Tween 80
754 desorbed 5.8% and 2.1% of phenanthrene and pyrene from soil into aqueous phase,
755 respectively; addition of both Tween 80 and DOM derived from pig manure compost
756 and pig manure further enhanced the desorption of phenanthrene to 15.8% and 16.2%,
757 respectively, and 6.4% and 10.9%, respectively, for pyrene. The authors found also that
758 the addition of mixed DOM–Tween 80 solution into the soil–water system enhanced
759 PAHs desorption, the enhancement effect being more than the additive effect of the
760 Tween 80 and DOM individually [169]. In general, this kind of mixed-type surfactant
761 systems may improve the performance of surfactant-assisted bioremediation of soils
762 and sediments by decreasing the applied surfactant level and thus remediation cost.
763 Presently, no report is available in the literature on the effects on mixed surfactants on
764 the solubilisation and/or biodegradation of B[a]P in soils and sediments.
765
766 **4.2. Cyclodextrin-based techniques**
767 The ability of cyclodextrins to complexed HOCs is well-known and has been widely
768 utilised for decades in the industries like pharmaceutical, food, cosmetic and
769 agriculture, their utilisation in environmental decontamination is more recent.
770 Traditionally, soil flushing with surfactants has been used to improve bioavailability of
771 HOCs in oil-contaminated soils and sediments [42,131,170-173]; however, in the last
772 decade interest is shifting to the use of cyclodextrins because they have negligible
773 surface reactivity, which minimises the adherence of entrapped contaminants to soil
774 particles in addition to eliminating the challenge of maintaining CMCs of surfactants
775 [174-178]. Also unlike surfactants, cyclodextrins have been shown to exhibit no or
776 negligible toxic effects on soil microbial ecology [116,175,179,180].

777 Cyclodextrins are typical toroid-shaped cyclic non-reducing oligosaccharides with six
778 (α-cyclodextrin), seven (β-cyclodextrin), or eight α-D-glucopyranose units (γ-
779 cyclodextrin) derived from starch [181]. Cyclodextrins form host–guest inclusion
780 complexes with lipophilic molecules through their hydrophobic central cavity; the
781 hydrophilic exterior is responsible for the large water solubility of the stable aqueous
782 inclusion complexes [181-183]. Cyclodextrin chemistry with the various host–guest
783 inclusion phenomena (e.g., inclusion complexation and encapsulation interaction) has
784 been comprehensively reviewed elsewhere [181]. A prerequisite for the formation of
785 inclusion of complexes and thus for an efficient extraction is that the size and shape of
786 the target molecules should fit into the cyclodextrin cavity, which increases from α-
787 (diameter 4.7–5.3 Å), to β- (6.0–6.5 Å), and to γ-cyclodextrins (7.5–8.3 Å). Examples
788 of synthetic cyclodextrins are 2-hydroxypropyl-β-cyclodextrin (HP-β-CD), methyl-β-
789 cyclodextrin (M-β-CD) and sulphate-β-cyclodextrin (S-β-CD).
790 A large body of studies has evidenced significant improvement in the apparent
791 solubilisation of PAHs by cyclodextrins [e.g. 180,184]. The aqueous solubilisation
792 capacity of cyclodextrins towards PAHs depends of a number of factors and
793 conditions, and in general, increases linearly with the cyclodextrin concentration. The
794 comparison of data reported in the literature indicates that in addition to the significant
795 influence of soil type, the molecular weight and structure of target PAHs as well as
796 their concentrations, and co-contaminant(s) type and concentration affect the extent to
797 which a given cyclodextrin solubilises the PAHs. For instance, there is a distinction in
798 cyclodextrin solubilisation capacity for PAHs in freshly-contaminated soils and aged-
799 contaminated soils [134, 174].
800 In a study by Viglianti et al. [185], three cyclodextrins, βCD, HP-β-CD and M-β-CD
801 were showed to be effective flushing-agents of PAHs from a soil column with M-β-CD

802 producing the highest removal efficiency followed by HP-β-CD and lastly βCD.
803 Temperature in the range of 5 to 35 °C had no significant influence; cyclodextrin
804 concentration and ratio of soil to washing solution were found to have a significant and
805 almost linear effect on PAH removal from a contaminated soil [185]. Ramsay et al.
806 [186] reported that HP-β-CD and M-β-CD enhanced the solubility of the individual
807 PAHs (naphthalene, acenaphthalene, phenanthrene, anthracene, pyrene or
808 fluoranthene) to similar extents while S-β-CD only slightly improved solubility of any
809 of the PAHs tested. PAH solubility also increased as concentration of HP-β-CD or M-
810 β-CD increased. The solubility of phenanthrene and pyrene was enhanced 40–50 times
811 in 5% (w/v) HP-β-CD while that of naphthalene, acenaphthalene, anthracene, or
812 fluoranthene was enhanced only 4–13 times [186]. These differences may be due to
813 stereoselective interactions and hydrogen bonding between a given cyclodextrin
814 structure and the individual PAH [187]. In a study to assess the effect of organic
815 amendments on the chemical extractability of HOC residues that had been present in
816 soils for more 12 years, Doick et al. [188] reported that the addition of HP-β-CD did
817 not result in a significant increase in chemical extractability after a 36-day contact time.
818 Cyclodextrins have been modified to increase their solubilisation capability. The
819 esterification of βCD with 3-(dodec-2-enyl)-dihydrofuran-2,5-dione produced
820 modified-βCDs, which exhibit amphiphilic properties and are highly efficient at
821 diminishing the surface tension of water and water–dimethysulfoxide (DMSO)
822 solutions, forming aggregates at very low concentration [126]. The ability of these
823 modified-βCDs to enhance PAH biodegradation has not yet been reported.
824 Several investigators have reported significantly improved PAH degradation rates
825 when supplemented with HP-β-CD [174,180,184,189-191]. Few studies have reported
826 no or marginal effects [143,192-194], and none to date have reported negative effects

827 on PAH biodegradation. Several studies that have reported HP-β-CD-enhanced
828 solubilisation and biodegradation of PAHs were focussed mainly on the LMW-PAHs
829 with relatively fewer studies on PAHs with four or more benzene rings [184]. Most of
830 these studies are usually been carried out in liquid media with isolated pure or mixed
831 cultures whose degradative properties have been enhanced *in vivo* [184,194]. HP-β-CD
832 has been shown to mobilise HOCs loosely-bound to SOM, making them more
833 bioavailable for biodegradation by soil microorganisms [174,180,185,193]. The
834 influence of HP-β-CD on bioavailability and biodegradation of phenanthrene
835 [191,193,195], pyrene [184], B[a]P [192,196] or hexadecane [193] has been
836 investigated in soils spiked with the individual PAH, or in historically contaminated
837 municipal gas plant site soils [174]; with rather inconsistent conclusions.
838 Both HP-β-CD and β-CD enhanced the apparent solubility and biodegradation of
839 phenanthrene dissolved in two NAPLs, hexadecane and di-2-ethylhexyl phthalate
840 (DEHP) [197]. Solubilisation of phenanthrene increased with increase in cyclodextrin
841 concentration, and the apparent concentration of phenanthrene in the hexadecane–water
842 system increased as twice as that in the DEHP–water system. The extent of
843 cyclodextrin-enhanced phenanthrene biodegradation was influenced by the type and
844 concentration of cyclodextrin as well as the type of NAPL used; extent of
845 biodegradation increased linearly with β-CD concentration from 0.72 mM to 7.2 mM
846 while increasing HP-β-CD concentration beyond 3.6 mM did not result in further
847 biodegradation [197].
848 While there are numerous studies on cyclodextrin-mediated biodegradation of PAHs
849 with many reporting positive results, in terms of both increased extent of degradation
850 and reduced toxicity after remediation for LMW-PAHs, only a handful laboratory
851 studies have been carried out for HMW-PAHs [143,184,194]. To date no published

852 studies have reported any significant improvement in cyclodextrin-aided
853 biodegradation of B[a]P. The addition of solubilising agents, such as Tween 80 or
854 cyclodextrin, to the incubation medium did not enhance the biodegradation of B[a]P by
855 *S. paucimobilis* EPA 505 [194]. Cuypers et al. [143] studied the enhancement effect of
856 HP-β-CD (applied at 0.18 and 0.79 g kg^{-1}) on PAH biodegradation in two MGP
857 sediments. While biodegradation enhancement was significant only for chrysene at the
858 higher HP-β-CD concentration in the first sediment, and for phenanthrene,
859 fluoranthene, benzo[a]anthracene, chrysene, and indeno[1,2,3-*c,d*]pyrene at the lower
860 concentration, and for benzo[g,h,i]perylene and indeno[1,2,3-*c,d*]pyrene at the higher
861 [1,2,3-*c,d*]pyrene concentration in the second sediment, overall enhancement effect of
862 HP-β-CD was negligible [143]. Hence, the authors concluded that HP-β-CD addition
863 neither stimulated nor inhibited PAH biodegradation in coal tar-contaminated soil. By
864 contrast, Triton X-100 did enhance PAH biodegradation in both sediment samples.
865 Recently, we evaluated the ability of HP-β-CD to enhance B[a]P solubilisation, and
866 hence, its biodegradation in unexposed and diesel oil-exposed soils (unpublished data;
867 Paper VII). It was found that rather than improving B[a]P mineralisation by indigenous
868 soil microorganisms, HP-β-CD significantly reduced mineralisation in three of the four
869 soils investigated. This was in spite of the significant improvement in the apparent
870 solubilisation of B[a]P in all the soils (Figure 2 adapted from Paper VII). In summary,
871 the results of these studies showed that presence of HP-β-CD may not enhance or may
872 even inhibit the degradation of PAHs, especially the HMW-PAHs like B[a]P, although
873 solubilisation of the pollutant was substantially increased by HP-β-CD. Reid et al.
874 [195] suggested that the risk posed by residual contamination is expected to be minimal
875 in HP-β-CD-aided biodegradation of phenanthrene in soil; this is yet to be test for
876 B[a]P.

877

878 **4.3. Co-solvent-aided techniques**

879 Non-biodegradable solvents have also been studied for their capacity to increase
880 pseudo-solubilisation of PAHs. The choice of a solvent as SEA is usually based on
881 environmental safety, ease of availability and cost, as compared to conventional
882 surfactants. Lee et al. [198] used soil pre-treatment with acetone and ethanol to
883 promote the biodegradation of PAHs in five coal tar-contaminated soils from former
884 MGP sites. The initial pre-treatment was found to accelerate the volatilisation of two-
885 and three-ring PAHs. The total PAH degradation rates for soils pre-treated with
886 solvents were estimated to be about two times faster than soils that were not pre-
887 treated. In particular, solvent pre-treatment enhanced removal of the carcinogenic five-
888 ring PAHs such as B[a]P, and to a limited extent the four-ring PAHs such as chrysene.
889 High organic carbon content was shown to have no effect on the co-solvent-aided
890 biodegradation, and there was no significant difference between acetone-treated and
891 ethanol-treated soils [198].

892 Villemur et al. [199] demonstrated an enhancement of the biodegradation of HMW-
893 PAHs including B[a]P in soil using two-liquid-phase (TLP) slurry bioreactors.
894 Addition of a water-immiscible, non-biodegradable, and biocompatible liquid, silicone
895 oil, to soil-slurry promoted desorption of PAHs from a field-contaminated soil. The
896 author attributed the initial rapid desorption during the first 8 h to the extraction of non-
897 solubilised and of poorly sorbed PAHs, and the later slower but constant transfer was
898 attributed to extraction of more tightly bound PAHs. The ability of a HMW-PAH-
899 degrading consortium to access and degrade the desorbed PAHs in the TLP soil-slurry
900 was compared to the control soil-slurry without silicone oil [199]. Pyrene degradation
901 was low, and no feasible biodegradation of chrysene and B[a]P observed in the control

902 slurry bioreactor. Pyrene was completely degraded after 4 days, and substantial
903 biodegradation of chrysene and B[a]P recorded in the TLP soil slurry bioreactor with
904 the PAH-degraders being more effective at degrading PAHs in the organic solvent
905 interface than in the aqueous medium [199]. Other non-biodegradable NAPLs, such as
906 2,2,4,4,6,8,8-heptamethylnonane (HMN), paraffin oil, hexadecane and corn oil were
907 much less, or not efficient in improving PAH degradation by the consortium; the
908 addition of surfactants (Triton X-100, Witconol SN70, Brij 35 and rhamnolipid), or
909 Inipol EAP22 also did not promote biodegradation [200]. Meanwhile, HMN increased
910 solubility and biodegradation of naphthalene and phenanthrene by *Pseudomonas* sp.
911 strain 9816/11 and *Sphingomonas yanoikuyae* B8/36 in aqueous solution [128]. These
912 contrasting findings indicate that the ability of chemical co-solvents to enhance
913 biodegradation depends on the solvent type and the microorganisms involved.
914 Surfactants have been used to stabilise the emulsion formed by solvents in order to
915 further enhance PAH solubilisation. Kim et al. [201] reported that the combination of
916 paraffin oil and Brij 30 increased the mobilisation through soil column and enhanced
917 biodegradation of phenanthrene than in water phase or Brij 30 solution only. On the
918 other hand, chelating or complexing reagents (e.g. citric acid or EDTA) with the ability
919 to increase solubility of SOM have been used facilitate the desorption of PAHs from
920 the particulate organic matter fraction into surfactant solution [202,203]. An added
921 advantage of using ethylenediaminetetraacetic acid (EDTA) or its structural isomers
922 like EDDS (ethylenediaminedisuccinic acid) is that the chelating agents have high
923 complexing capacity to mobilise metal cations coupled with only a minor impact on
924 physical and chemical properties of the soil matrix [202]. Wen et al. [203]
925 demonstrated significantly high mobilisation of B[a]P and chrysene following

926 ultrasonic mixing of field-contaminated soil with a combination of 20 ml l^{-1} surfactant
927 suspension and a sparing quantity (2 mmol) of [S,S]-stereoisomer EDDS.
928 Other studies have used natural or plant-derived oils as alternative to the chemical
929 solvents due to their being non-toxic, more cost-effective and biodegradable.
930 Sunflower oil was reported to enhance the mobilisation of PAHs from a heavily-
931 contaminated soil [204]. Recently, Lladó et al. [93] reported a significantly higher
932 B[a]P degradation and one order of magnitude increase in bacterial heterotrophic
933 population in sunflower oil-amended soil compared to the same soil amended with Brij
934 30 or with no SEA addition. In addition to the possibility of acting as extra partition
935 phase, sunflower oil supplies readily accessible and degradable carbon substrates to
936 enhance the cometabolism of B[a]P.
937
938 **4.4. Other natural/biogenic SEAs**
939 It is well accepted that PAHs interactions with natural organic matter (NOM) in soils
940 and sediments influence the pollutants solid–liquid distribution and transport –
941 mechanisms which define their bioavailability, and to a large extent, their
942 biodegradability in soils and sediments [205-207]. NOM including that which may be
943 present in solid-phase (NSOM) or in dissolved form (NDOM), can act as a sink or a
944 mobilising agent, respectively [106,208]. In soil environments, the reduced
945 bioavailability of PAHs due to sorption to NSOM is an important factor controlling
946 their biodegradation, and several studies have evidenced an inhibitory effect of NSOM
947 on the biodegradation of PAHs in soils and sediments [205,209-212]. While studies
948 have reported variable effects of NDOM on biodegradation of HOCs, there is an
949 increasing body of evidence pointing to an enhancing effect in the case of PAHs [106].

950 The mechanism(s) of the mobility enhancement are not yet well understood. However,
951 it is generally thought that naturally-occurring amphiphiles, such as dissolved humic
952 substances, do not form micelle-like aggregates like the surfactants or inclusion
953 complexes like the cyclodextrins; a macromolecular model has been suggested [178].
954 A number of mechanisms have been proposed for NDOM-mediated biodegradation of
955 HOCs: (i) a result of enhanced desorption of HOCs from soils [213]; (ii) a direct access
956 to DOM-sorbed PAHs due to the physical association of bacteria and DOM [214]; and
957 (iii) an increased diffusive flux toward bacterial cells cause by DOM [215].
958 A commercially available natural polymer (Salmon deoxyribonucleic acid, DNA),
959 which was successfully applied to the flushing of PAH-contaminated soil, has been
960 described as environmentally safe and non-exhaustive, and with large inclusion sites
961 that accommodate even HMW-PAHs, and has a high specificity to PAHs and other
962 planar aromatic organic compounds [216]. In a spiked soil that contained 72 mg kg^{-1}
963 anthracene, 102 mg kg^{-1} phenanthrene, and 99 mg kg^{-1} pyrene, extractions close to 88,
964 78 and 94%, respectively, were attained with 5% DNA at 1:50 soil/extractant ratio
965 [216]. Compared to Tween 80, β-CD and M-β-CD, this natural SEA also exhibited
966 greater solubilisation capacity toward HMW-PAHs like pyrene [216]. In another study,
967 it was reported that the degradability of different PAHs including anthracene,
968 phenanthrene and pyrene by *Sphingomonas* sp. was not inhibited even at a high DNA
969 concentration of 2%, and that DNA was stable against the PAH degrader, suggesting
970 that a structural change in the polymer is not necessary for the release of PAHs [217].
971 Degradation of pseudosolubilised B[a]P by *Sphingomonas* sp. following an initial
972 dissolution of the compound in 1% aqueous DNA solution was demonstrated to be
973 significantly greater (95%) compared to the PAH crystals in aqueous medium (40%);
974 this indicates that the intercalation-like binding of the PAHs in the polymer does not

975 pose serious constraint to bacterial uptake [218]. The performance of hexane
976 regenerated DNA was stable after three to four stages of recycling [216].
977 Kobayashi et al. [219] reported a significant increase in pyrene desorption from soil
978 amended with DOM extracts derived from a mixed gardening compost or cow manure,
979 and showed that the application of the DOM extracts to a PAH-contaminated soil
980 enhanced the phytoremediation by two subspecies of *Cucurbita pepo*. In another study,
981 Kobayashi et al. [220] investigated the effect of water-extractable organic matter
982 (WEOM) from manure compost of cow, chicken and pig on the biodegradation of
983 various PAHs. WEOM significantly increase the apparent dissolution of phenanthrene,
984 pyrene, and B[a]P and enhanced the biodegradation of the PAHs in liquid medium.
985 B[a]P degradation by *Sphingomonas* sp. was enhanced by almost 3-times with the
986 extent increasing from 25.1% in the absence to 73.1% in the presence of WEOM after
987 14 days. The authors observed that WEOM with molecular mass >1000 Da mainly
988 contributed to the solubility and biodegradation enhancements [220].
989 Berselli et al. [189] compared the effects of surfactants, cyclodextrins, humic
990 substances and rhamnolipids on desorption of HOCs including PAHs from an aged
991 contaminated soil and on the biodegradation of resulting effluents. The capability of
992 water to elute the HOCs was significantly enhanced with the biogenic SEAs (by 237%,
993 βCD; 265%, HP-β-CD; 400%, rhamnolipids; 566%, humic substances) and with
994 synthetic surfactant Triton X-100 (660%). Triton X-100 recorded the lowest depletion
995 of the initial soil ecotoxicity, the greatest impact on the soil organic matter, as well as
996 adversely affected the bioremediation of the resulting effluent by inducing a premature
997 decrease of specialised bacterial biomass. By contrast, the biogenic SEAs, and in
998 particular rhamnolipid and humic substances, sustained the biodegradation and

999 dechlorination of pollutants by apparently enhancing the availability of specialised
1000 bacteria in the reactors [189].
1001 Berselli et al. [221] reported on the development of an innovative soil-washing process
1002 using cheap, non-toxic, and biodegradable SEAs including deoxycholic acid (DA),
1003 bovine bile (BB), and the residue resulting from DA extraction from BB (BBR). These
1004 biogenic SEAs enhanced the mobilisation of pollutants from a soil historically
1005 contaminated with chlorinated anilines and benzenes, thiophenes, and several PAHs by
1006 230–440%, as compared to 540% in the case of the synthetic surfactant Triton X-100.
1007 However, the biogenic SEAs, and in particular DA and BB, mediated greater
1008 depletions of the initial soil ecotoxicity and enhanced the effluents biodegradation by
1009 sustaining the growth and increasing complexity of the effluent eubacterial
1010 communities; on the contrary, Triton X-100 adversely affected the bio-treatability of
1011 the resulting effluents [221].
1012 Fava et al. [222] demonstrated the ability of soya lecithin (SL) and humic substances
1013 (HS) applied at 1.5% (w/w) to a soil historically contaminated with a large variety of
1014 PAHs (13 g kg^{-1}) to enhance mobility and biodegradation in aerobic solid-phase and
1015 slurry-phase reactors. A slow and partial biodegradation of LMW-PAHs along with a
1016 moderate depletion of the initial soil ecotoxicity was observed in the control reactors.
1017 By contrast, the overall removal of PAHs was faster and more extensive and
1018 accompanied by a larger soil detoxification in soils amended with either SL or HS,
1019 especially in slurry-phase conditions. The authors concluded that SL and HS enhanced
1020 PAH mobility to the water phase, as well as serve as substrates for increase growth of
1021 indigenous aerobic PAH-degrading bacteria [222].
1022 Other biogenic surfactants including those synthesised from organic wastes such as
1023 potato starch [223] have been investigated for their ability to improve solubilisation of

PAHs. Rosu et al. [223] demonstrated that, in comparison with the native potato, all the alkylated starches (including those with epoxyalkane, alkenyl succinic anhydride and 1,4-butane sulfonone) showed an enhancement of their aqueous solubility with B[a]P aqueous solubilisation being significantly stimulated by the ester-modified starches. However, the capacity of these biogenic SEAs to enhance PAH biodegradation is yet to be reported.

5. Limitations to SEA-assisted B[a]P biodegradation in soil and sediments

Studies on SEA-assisted biodegradation, especially of the LMW-PAHs, in soils and sediments present widely varying and conflicting reports [125,142,147,224]. The effects range from enhancements, to no-effect at all, and to inhibitions of PAH biodegradation. The appraisal of these confounding results indicates the influence of a variety of factors ranging from the SEA type and concentration, to PAH type and concentration, to PAH-degrader type, and to soil characteristics as well as experimental conditions [116,225,226]. Basically, the application of SEAs in bioremediation of PAH-contaminated soils and sediments is aimed at achieving an increase in the pseudosolubilisation of the otherwise insoluble PAHs, and possibly an increase in the microbial growth by serving as alternative carbon substrate. The ability of SEAs to increase PAH solubilisation is largely dependent, among other parameters, on the presence, type and concentration of co-contaminants – factors which may not be favourable to B[a]P, and the SEA-stimulated microbial growth is not known to be selective for PAH degraders in the heterotrophic microbial community. Therefore, the effectiveness of this technology in bioremediation of B[a]P may remain fragmentary and unpredictable under field conditions.

In general, the solubilisation enhancement capacity of most surfactants and cyclodextrins tends to be greater than their biodegradation enhancement capacity. An evaluation of studies that have reported SEA-enhanced biodegradation of PAHs indicates that, in most cases, enhancements did not correlate well with extents of PAH solubilisation, suggesting other physiological and physical–chemical influences of the SEAs on microbial activity. For instance, surfactants, at concentrations above their CMCs, hardly fail to improve PAH solubility in water but are inconsistent at enhancing PAH biodegradation. Studies have shown positive, negative, or no-effects of surfactants on the abundance and diversity as well on the growth of indigenous microorganisms in soil and sediments.

The negative effects of surfactants on PAH biodegradation may be as a result of their toxicity to microorganisms or the increased toxicity of PAHs due to their greater bulk concentration in the micelles [130]. In addition, the limitation of cells to access pseudosolubilised PAHs in the micellar-phases of surfactants or inclusion complexes of cyclodextrins, and the preferential utilisation of these SEAs by PAH-degrading microorganisms result in reduced biodegradability. As PAH-contaminated soils and sediments are prone to a higher C:N:P ratio, the introduction of surfactants with large proportion of readily available and degradable C substrate may further markedly increase the C:N:P balance, leading to greater nutrient limitations as biodegradation proceeds. This may increase the cost of remediation through the addition of extra N and P supplements. As surfactants and cyclodextrins are relatively more biodegradable than PAHs, the applications of these SEAs to PAH-contaminated soils may alter the succession in the microbial community responsible for biodegradation [227]. SEAs may also be a competing substrate, affecting negatively PAH biodegradation kinetics [125,142].

As observed by Kim and Weber [228] in a study that investigated the biodegradation of phenanthrene in the presence of the nonionic Tween series surfactants, the PAH-degrading strain *Sphingomonas paucimobilis* EPA 505 preferentially utilised the surfactants, resulting in the destabilisation of surfactant micelles and the re-crystallisation of the initially dissolved phenanthrene molecules. Meanwhile, it was initially observed that dosages of surfactants in excess of their respective CMC dramatically enhanced solubilisation of phenanthrene. If these occurred during B[a]P biodegradation, the increased toxicity as a result of instantaneous dissolution of B[a]P may change the microbial community and adversely affect its degradative ability, greater mobilisation and bioaccessibility of the pseudo-solubilised B[a]P may increase toxicity to other sensitive soil biota populations, and partial or preferential utilisation of surfactants may revert associated B[a]P to a crystallised form without biodegradation. In a case that the surfactants and B[a]P are competitively degraded, partial transformation of the parent B[a]P to its more water soluble and genotoxic diol metabolites will actually present greater risks.

In a similar manner, recent unpublished research from the authors' laboratory, which investigated the effect of the presence and concentration of HP-β-CD highlighted that while B[a]P apparent solubility increased, the extent of mineralisation decreased as HP-β-CD concentration increased from 12.5 mM to 50 mM (Paper VI). Further, biodegradation of B[a]P by indigenous soil microorganisms was adversely affected in three of the four soils investigated, resulting in up to 25-times decrease in the extent of mineralisation (Paper VII). In all of these scenarios, deployment of SEA-aided bioremediation will be more detrimental to the environment and may lead to higher remediation costs. Unfortunately, no study has investigated the ecotoxicity effects on sentinel soil biota following SEA-assisted B[a]P biodegradation.

1098 Although cyclodextrins on their own are not known to exhibit negative effects on
1099 microbial survival and growth; however, a number of studies have reported increased
1100 toxicity to microorganisms due to the instantaneously-desorbed bulk concentrations of
1101 PAHs in cyclodextrin solution. In addition, and similar to the effect of surfactants,
1102 biodegradation of cyclodextrins may result in the rapid depletion of essential nutrients
1103 and oxygen, causing reduced microbial activity or slower PAH degradation rate.
1104 Whereas, most non-biodegradable co-solvents are not toxic to microorganisms and
1105 may not compete with PAHs as alternate carbon source, *in situ* application of non-
1106 biodegradable co-solvents to contaminated soils and sediments is not environmentally
1107 friendly, and hence not advisable.
1108 Recycling of the surfactants is desirable to decrease remediation costs; however in
1109 practice, it is difficult to separate pseudo-solubilised contaminants from surfactants and
1110 the often too quick biodegradation of surfactants could make the recovery and reuse to
1111 have little effect on remediation costs. No study has attempted to recover cyclodextrins
1112 following PAH degradation in soil as to assess the feasibility of their reuse.
1113 It has to be mentioned also that due to the differences in their physical-chemical
1114 characteristics, the limitations to SEA-assisted biodegradation may be different in soils
1115 and sediments. Sediments are heterogeneous materials which are preponderantly fine
1116 particles with a higher proportion of organic matter and water compared to soils. Soil
1117 texture, organic matter and clay contents, water level have all been shown to affect the
1118 capacity of SEAs to desorb and/or solubilise PAHs.
1119 Another undesirable effect of the application of SEAs is that rather than enhancing the
1120 solubilisation of PAHs, surfactants may promote increased sorption to soil matrix. This
1121 is especially a major limitation to the applicability of nonionic and cationic surfactants
1122 to enhance bioremediation in aged PAH-contaminated soils. Studies have reported

1123 enhanced sorption of PAHs to soils in the presence of immobile adsorbed surfactants
1124 [124,177]. This phenomenon may increase the risk associated with the surfactant-
1125 assisted bioremediation as sorbed contaminants may remain available to other sensitive
1126 non-microbial receptors. This also raises a concern about the fate of bound residues
1127 after microbial degradation has ceased in a contaminated soil; the remobilisation of the
1128 sorbed contaminants may represent a new risk.
1129 The type and concentration of the SEAs may have significant influence on the
1130 effectiveness of SEA-assisted bioremediation. As many sites contaminated with crude
1131 or refined petroleum oils are characterised by the presence of complex mixtures of
1132 HOCs and metals, and with the high variability in soil characteristics, the efficiency of
1133 these SEAs to enhance the biodegradation of B[a]P may be limited. For example,
1134 cyclodextrins are capable of forming various types of host–guest complexes by
1135 selectively incorporating PAHs through size and polarity considerations [229,230]; a
1136 phenomenon which favours the LMW-PAHs more and could affect the amount of
1137 B[a]P desorb and/or solubilised in field-contaminated soils and sediments.
1138 Overall, synthetic surfactants are able to increase the apparent solubilisation of B[a]P,
1139 but there is doubt that they can enhance its biodegradation in soils or sediments. The
1140 information available in the literature is sparse and inconsistent, current evidence does
1141 not yet supports the enhancements of solubilisation and/or biodegradation of B[a]P in
1142 the presence of biosurfactants. Likewise, natural amphiphiles, like DOM, may increase
1143 solubilisation but their capacity to substantially enhance B[a]P requires further
1144 investigations. A sizeable body of evidence has shown that cyclodextrins are able to
1145 significantly increase the desorption and apparent solubilisation of PAHs; apparent
1146 solubilisation of B[a]P up to four-order in water and up to two- to three-order of
1147 magnitudes in soils has been reported. Presently however, the evidence for

1148 cyclodextrin-based B[a]P biodegradation is inconclusive, albeit, it appears to tend
1149 towards inhibition.
1150 Most of the published laboratory studies on SEA-assisted biodegradation of PAHs are
1151 conducted under aerobic conditions, and involved using pure culture of individual or a
1152 mixture of few species of bacteria. Further research is needed to understand the effects
1153 of SEA-assisted biodegradation under anoxic/anaerobic conditions, which are common
1154 in sediments and shallow aquifers and subsurface areas in contaminated soils. Research
1155 to characterise and optimise parameters including the effects of soil physicochemical
1156 properties, type and concentration of co-contaminants (organic and inorganic),
1157 interacting and/or interfering naturally-occurring amphiphiles (e.g. humic acids),
1158 nutrient availability, and salinity should be carried out, especially with HMW-PAHs.
1159 Obviously, a concerted research focus is required to investigate SEA-assisted
1160 biodegradation of B[a]P in soils and sediments. There is also the need to further
1161 investigate the risks associated with SEA-assisted bioremediation at the field-scale.
1162 Evidence from the literature highlights the need to always evaluate the effects of SEAs
1163 on the catabolic activity of the autochthonous microbiota in polluted soils before
1164 scaling up remediation process at field scale. In addition, the toxicity to microbial
1165 community and other environmental receptors due to the SEA itself, or that resulting
1166 from increased solubilisation of PAHs or their metabolites, as well as toxicity induced
1167 by nutrient depletion as a result of rapid SEA biodegradation should be considered. The
1168 compounding influences of edaphic factors, such as availability of nutrients, energy
1169 sources and electron acceptors, types and concentrations of co-contaminants, types and
1170 concentrations of toxicants, spatial and temporal variability in pH, temperature,
1171 moisture content, dissolved oxygen, and soil texture and organic matter and clay
1172 contents of the contaminated soil should be taken into consideration when assessing

1173 potential SEAs to assist in the biodegradation of target PAHs. The complex influences
1174 of wide-ranging climatic conditions and overall hydrogeology of the contaminated sites
1175 should also be taken into account. Because these factors are often site-specific and are
1176 highly variable in terms of space and time, engineering models need to be attuned
1177 accordingly when designing a remediation strategy. The engineering requirements,
1178 economic and time costs, as well as the ecological and ecotoxicological impacts on
1179 other environmental receptors should also be careful considered on a case by case
1180 basis. The challenge yet remains of translating the well-controlled laboratory research
1181 results to field applications where edaphic and environmental factors are so variable
1182 and dynamic.
1183
1184 **6. Concluding remarks**
1185 Pollution of the environment with PAHs has enormous socio-economic, ecological and
1186 ecotoxicological (including human health) impacts, hence the need to intensify
1187 research into detoxification of polluted soil and sediment remains critical to sustainable
1188 development. A diverse range of microorganisms has been reported able to degrade
1189 PAHs and/or detoxify contaminated soils and sediments. However, physicochemical
1190 properties of PAHs and their interactions with soil particulate matters, as well as
1191 certain environmental factors can influence the ability of microorganisms to access
1192 PAHs in soils and sediments. The inability of surfactants to enhance the biodegradation
1193 of PAHs, especially those with greater than three-rings, has been attributed to a number
1194 of factors, including surfactant toxicity, poor bioavailability of the micellar or
1195 complexed PAHs, inhibition of bacterial attachment, increased PAH sorption as a
1196 result of surfactant sorption, nutrient deficiency resulting from surfactant

biodegradation, in addition to poor microbial catabolic activity due to effects on the development of a microbial community unfit for PAH biodegradation.

Most of the published studies on SEA-assisted biodegradation in soil or sediment ecosystems assayed for the LMW-PAHs wherein the challenge of both microbial degradative capacity and mass transfer may not be present at the same time, as in the case of B[a]P. The capacity of SEAs to improve B[a]P solubilisation (i.e., desorption from solid phase or dissolution from NAPL phase to the aqueous phase) in contaminated soil and sediment is certainly not in doubt; what remains questionable, based on available literature, is their ability to actually increase biodegradation of PAHs for greater bioremediation efficiency.

To date, information available in the literature on SEA-assisted B[a]P biodegradation is rather limited and contradictory, making feasibility assessment of the applicability of this technology to bioremediation of soil or sediment with high burdens of the potent carcinogen, B[a]P a challenge. Therefore, before a decision is made on the application of SEA-assisted B[a]P biodegradation in soils and sediments, there is need to establish that this technology is deemed the best practicable environmental option (BPEO) and/or best alternative technology not entailing excessive cost (BATNEEC).

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Tables

Table 1: Physicochemical properties of priority PAHs [38,18,55].

PAH	Abr.	NR[a]	MW[b]	CF[c]	mp[d]	bp[e]	Sol[f]	K_{ow}[g]	V.P.[h]
Naphthalene [NC]	Nap	2	128.2	$C_{10}H_8$	79–82	218	32.0	3.37	10.9
Acenaphthene [NC]	Acy	2	152.2	$C_{12}H_{10}$	95	265–275	5.30	3.94	5.96×10^{-1}
Acenaphthylene [NC]	Ace	2	154.2	$C_{12}H_{18}$	72–82	96.2	3.93	4.07	5.96×10^{-1}
Fluorene [NC]	Flu	2	166.2	$C_{13}H_{10}$	115–116	295	1.85	4.15	8.86×10^{-2}
Phenanthrene [NC]	Phe	3	178.2	$C_{14}H_{10}$	99	340	1.29	4.22	1.8×10^{-2}
Anthracene [NC]	Ant	3	178.2	$C_{14}H_{10}$	218	340	0.64	4.41	2.0×10^{-4}
Fluoranthene [NC]	Fla	3	202.3	$C_{16}H_{10}$	110	~375	0.25	4.74	2.54×10^{-1}
Pyrene [NC]	Pyr	4	202.1	$C_{16}H_{10}$	156	360	0.14	4.82	8.86×10^{-4}
Benzo[a]anthracene [C]	B[a]A	4	228.3	$C_{18}H_{12}$	158	400–435	0.01	5.25	7.3×10^{-6}
Chrysene [WC]	Chr	4	228.3	$C_{18}H_{12}$	255	488	0.002	5.61	5.7×10^{-7}
Benzo[*b*]fluoranthene [C]	B[b]F	4	252.3	$C_{20}H_{12}$	168	–	–	6.11	–
Benzo[*k*]fluoranthene [C]	B[k]F	4	252.3	$C_{20}H_{12}$	215	480	–	6.11	5.7×10^{-8}
Benzo[*a*]pyrene [SC]	B[a]P	5	252.3	$C_{20}H_{12}$	179	496	0.0038	6.04	1.5×10^{-5}
Dibenz[*a,h*]anthracene [C]	D[ah]A	5	278.4	$C_{22}H_{14}$	273	–	0.0005	6.84	1.8×10^{-6}
Benzo[*g,h,i*]perylene [NC]	B[ghi]P	6	276.4	$C_{22}H_{12}$	262	550	0.00026	6.20	2.0×10^{-5}
Indeno[1,2,3-*c,d*]pyrene [C]	InP	6	276.3	$C_{22}H_{12}$	163	536	0.062	7.66	–

[a] NR: Number of aromatic rings; [b] MW: molecular weight (g mol^{-1}) [c] CF: chemical formula; [d] mp: melting point (°C); [e] bp: boiling point (°C);
[f] Sol: aqueous solubility (mg l^{-1}); [g] log K_{ow}: logarithm of the *n*-octanol–water partitioning coefficient; [h] VP: Vapour pressure (Pa, 25 °C);
(NC): Non-carcinogenic; (C): Carcinogenic; (WC): Weakly-carcinogenic; (SC): Strongly-carcinogenic.

1907 Table 2: Concentration of US EPA priority PAHs in background soils across the world.

PAH	Bangkok[a]	UK[b]	Antarctica[c]	China[d]	India[e]	India[f]	Ghana[g]	Brazil[h]
	($\mu g\ kg^{-1}$ DW soil)							
Nap	1.7–145.2	ND–30.9	ND	10.8–33.1	99–706	3.0–30.0	5.0–24.0	1.1–356
Acy	0.1–6.1	ND–1.6	ND	ND–1.1	56–430	0.1–3.8	0.4–1.6	ND–2.0
Ace	0.1–4.0	ND–1.4	ND	ND–4.1	80–324	ND–3.1	0.4–0.8	0.1–2.2
Flu	0.2–4.5	0.8–1.6	1.16	0.9–4.2	50–154	0.2–3.9	1.3–2.4	ND–7.0
Phe	ND–60.8	18.9–34.0	1.13	2.8–16.7	38–191	0.6–48.0	7.3–11.0	0.23–36
Ant	0.1–5.0	9.9–16.9	0.66	0.9–14.2	13–109	ND–6.2	1.3–1.9	0.01–2.6
Fla	ND–45.8	0.4–6.7	ND	0.7–14.1	70–520	0.5–56.0	3.5–4.8	0.26–18.0
Pyr	0.3–48.3	1.7–2.5	0.69	0.4–7.7	51–267	0.4–39.0	3.1–4.1	0.17–14.0
BaA[C]	0.1–34.7	ND–3.3	1.47	0.7–5.8	29–83	0.1–25.0	0.9–1.1	ND–1.6
Chr[C]	0.3–29.7	0.6–1.1	0.32	0.8–12.5	18–143	0.3–32.0	2.7–3.5	0.05–4.7
B[b]F[C]	0.8–49.7	0.4–3.6	1.29	0.9–17.8	33–187	0.4–56.0	5.7–6.6	ND–5.2
B[k]F[C]	–	5.9–11.2	0.62	0.8–14.2	23–71	0.1–25.0	–	–
B[a]P[C]	0.2–22.3	ND–1.4	ND	ND–6.8	18–71	0.1–2.2	1.9–2.2	ND–1.9
D[ah]A[C]	ND–4.2	0.7–1.6	ND	ND–6.6	ND–276	0.1–14.0	0.2–0.5	ND–0.39
B[ghi]P	0.7–58.9	ND–0.4	ND	ND–4.9	ND–214	0.4–60.0	1.3–1.9	ND–4.7
InP[C]	0.4–28.4	1.1–8.0	0.53	ND–6.1	ND–226	0.2–64.0	2.2–2.7	ND–3.8
% B[a]P	4	0–1	0	0–4	2–3	0.5–2	3–5	0–0.5

1908 ND: Below detection limit
1909 [a] 20 different sites in Bangkok, Thailand [231]; $\sum$20 PAHs
1910 [b] 4 different soils from Lancashire county, UK (this thesis); $\sum$16 PAHs
1911 [c] Soils from the Antarctica (this thesis); $\sum$16 PAHs
1912 [d] 3 suburbs in China [232]; $\sum$16 PAHs
1913 [e] 7 different sites in Delhi, India [233]; $\sum$16 PAHs
1914 [f] Urban soils in India [35]; $\sum$20 PAHs
1915 [g] 4 rural agricultural and forest soils in Ghana [35]; $\sum$20 PAHs
1916 [h] 6 climatically different regions of Brazil [37]; $\sum$20 PAHs
1917

Table 3: Surfactant properties and PAH solubility [125].

Surfactant	Mol. wt. (Average)	HLB[a]	CMC (mM)	MSR[b]			log K_m[c]		
				Pyr	Fla	B[a]P	Pyr	Fla	B[a]P
Brij 35	1,200	16.9	0.0625	0.0570	0.0871	0.0198	5.54	5.68	7.78
Igepal CA-630	603	13.0	0.1078	0.0326	–[d]	–	5.39	–	–
Tergitol NP-10	652	13.1	0.0690	0.0336	0.0502	0.0118	5.71	5.56	7.43
Triton X-100	625	13.5	0.2320	0.0352	0.0508	0.0113	5.84	5.64	7.49
Tyloxapol	4,500	12.9	0.0167	0.0820	–	–	6.60	–	–

[a] Hydrophile–lipophile balance number. Data were obtained from the manufacturers; [b] The critical micelle concentration of each surfactant was determined in BSM at 30°C.
[c] The molar solubilisation ratio and micelle-phase/aqueous-phase partition coefficient were determined for pyrene (Pyr), fluoranthene (Fla), and benzo[*a*]pyrene (B[a]P) in BSM at 30°C.
[d] "–," not tested.

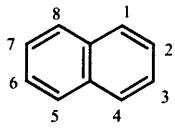
1924 Table 4: Effects of surfactants on PAH biodegradation [105].

Surfactant	Conc.	Target compounds	Conc. (mg/l)	Redox potential	Culture	Matrix	k^a	Performance	Reference
Tween 80	675× CMC	Naphthalene/ phenanthrene/ anthracene	500	Aerobic	*Pseudomonas* sp. *Enterobacter* sp. *Stenotrophomonas* sp.	Water phase	5.2×10^{-4}–2.0×10^{-2}	Greatly enhanced degradation	[234]
	19–112× CMC	Naphthalene, phenanthrene	10–110	Aerobic	Mixed	Soil slurry	5–31.2	Enhanced	[133]
	13–66× CMC	Phenanthrene	3.8–27.5	Aerobic	*Sphingomonas paucimobilis*	Water phase	0.066–0.091	Inhibitory effects	[137]
	73× CMC	Phenanthrene	27.8	Aerobic	Sphingomonas paucimobilis	Water phase	0.559	No degradation	[137]
	730–3,650× CMC	Phenanthrene	110–115[c]	Aerobic	*Sphingomonas paucimobilis*	Soil phase	0.283–0.304	Inhibit the degradation	[235]
	0.5 g kg^{-1}	Fluoranthene	200[c]	Aerobic	*Pseudomonas alcaligenes* PA-10	Soil phase	1.46	Enhance the degradation	[141]
	0–66× CMC	Phenanthrene	100	Aerobic	*Mycobacterium* spp. KR2	Solution with phenanthrene	0.018–0.115	Enhance the degradation	[123]
	1.5 g/l	11PAHs	5.5[c]	Aerobic	Sludge	Sludge phase	0.012–0.143	Enhance the degradation	[236]
	0–3× CMC	Phenanthrene	230	Thermophilic aerobic	*Bacillus* sp. B-UM	Water phase	0.006–0.504	Inhibit the degradation	[159]
Brij 30	26–155× CMC	Naphthalene, phenanthrene	10–110	Aerobic	Mixed	Soil slurry	10–44	No toxic effects	[133]
	0–93× CMC	Phenanthrene	NA	Aerobic	*Mycobacterium* spp. KR2	Solution with phenanthrene	NA	Enhance the growth of bacteria >40 mg/l inhibition	[123]
Brij 35	0–12× CMC	Phenanthrene	NA	Aerobic	*Mycobacterium* spp. KR2	Solution with phenanthrene	NA	Enhance the growth of bacteria >40 mg/l inhibition	[123]
10LE	0–14× CMC	Phenanthrene	NA	Aerobic	*Mycobacterium* spp. KR2	Solution with phenanthrene	NA	Enhance the growth of bacteria >40 mg/l inhibition	[123]
Triton X-100	73× CMC	Naphthalene/ phenanthrene/ anthracene	500	Aerobic	*Pseudomonas* sp. *Enterobacter* sp. *Stenotrophomonas* sp.	Water phase	5.2×10^{-4}–2.0×10^{-2}	Greatly enhanced degradation	[234]
	0–3× CMC	Phenanthrene	230	Thermophilic aerobic	*Bacillus* sp. B-UM	Water phase	0.005–0.504	Inhibit the degradation	[159]

	18–110× CMC	Naphthalene, phenanthrene	5–40	Aerobic	Mixed	Soil slurry	5.0–16.0	No toxic effects	[133]
	0.3–11.7× CMC	Naphthalene phenanthrene	5,000	Aerobic	*Pseudomonas* sp. *Sphingomonas yanoikuyae*	Micellar solution	Nap: <0.02[b]; Phe: 0.32–0.50[b]	Inhibitory effects	[128]
	5.8, 23.3× CMC	Naphthalene, phenanthrene, pyrene	21, 0.8	Aerobic	Mixed	Aqueous phase and micellar phase	7.2, 0.216	Increase the degradation rate	[237]
	0-1,000 mg/l	Phenanthrene	5.5c	Aerobic	Mixed	Soil	33.3	Increase the bioavailability	[238]
Tergitol NP-10	100 mg/l	Anthracene, pyrene	1.0	Aerobic	Mixed	Micellar solution	0.022	Negative impact	[138]
	230× CMC	Naphthalene/ phenanthrene/ anthracene	500	Aerobic	*Pseudomonas* sp. *Enterobacter* sp. *Stenotrophomonas* sp.	Water phase	5.2×10^{-4}–2.0×10^{-2}	Greatly enhanced degradation	[234]
Tergitol 15S7	3–19× CMC	Phenanthrene	0.70–2.13	Aerobic	*Neptunomonas naphthovorans*	Saline water	0.108–0.47	Inhibit at high concentration	[239]
LAS	0–2.1× CMC	Phenanthrene	NA	Aerobic	*Mycobacterium* spp. KR2	Solution with phenanthrene	NA	Slightly increased below 10 mg/l	[123]
TDTMA	0–9× CMC	Phenanthrene	100	Aerobic	*Mycobacterium* spp. KR2	Solution with phenanthrene	3.5	Toxic to bacteria	[123]
SDS–TW80	5.0–0.5; 2.0–0.5	Phenanthrene	27; 18	Aerobic	Mixed	Surfactant solutions	7.6–9.0	No toxic effect and enhance the degradation	[115]
SDS–Brij 35	5.0–1.0; 2.0–1.0	Phenanthrene	34; 25	Aerobic	Mixed	Surfactant solutions	8.6–12.0	No toxic effect and enhance the degradation	[115]
SDS–TX100	5.0–1.0; 3.0–1.0 mM	Phenanthrene	20; 15	Aerobic	Mixed	Surfactant solutions	6.86–10.3	No toxic effect and enhance the degradation	[115]
JBR rhamnolipid	0.5 gkg^{-1}	Fluoranthene	200[c]	Aerobic	*Pseudomonas alcaligenes* PA-10	Soil phase	0.1209	Enhance the degradation	[141]
P-CG3	0–3× CMC	Phenanthrene	230	Thermophilic aerobic	*Bacillus* sp. B-UM	Water phase	0.007–0.504	Inhibit the degradation	[159]
P9027	0–3× CMC	Phenanthrene	230	Thermophilic aerobic	*Bacillus* sp. B-UM	Water phase	0.005–0.504	Inhibit the degradation	[159]

NA not available; [a] k is the reported first-order kinetic rate constant; [b] Specific growth rate h^{-1}; [c] Unit: mg/kg

Figures



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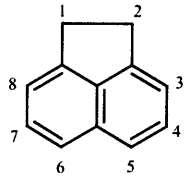

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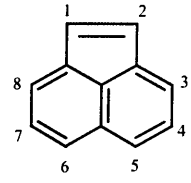
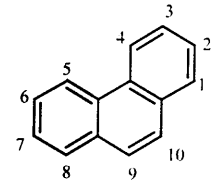


Acenaphthylene



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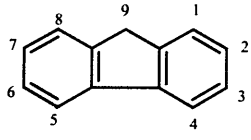
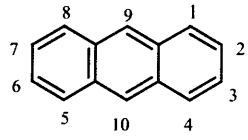


Fluorene



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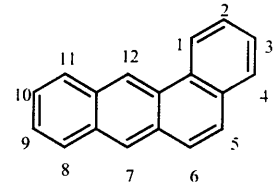
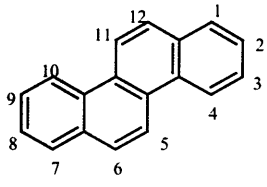


Benz[*a*]anthracene



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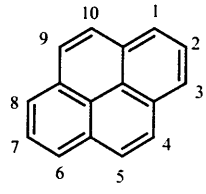
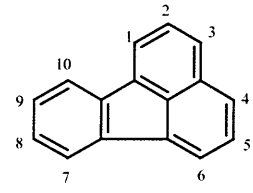
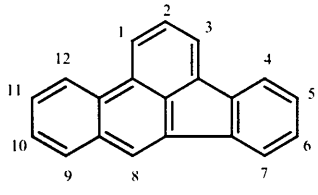


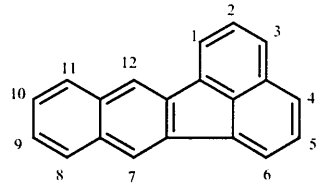
Pyrene



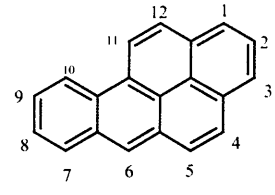
Fluoranthene



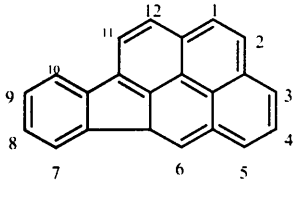
Benzo[*b*]fluoranthene



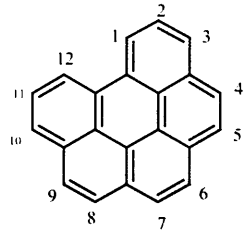
Benzo[*k*]fluoranthene



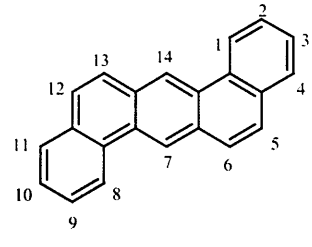
Benzo[*a*]pyrene



Indeno[1,2,3-*cd*]pyrene



Benzo[*ghi*]perylene



Dibenz[*a,h*]anthracene

Figure 1: Chemical structure and nomenclature of the 16 PAHs on the EPA priority pollutant list commonly encountered in the environment [2].

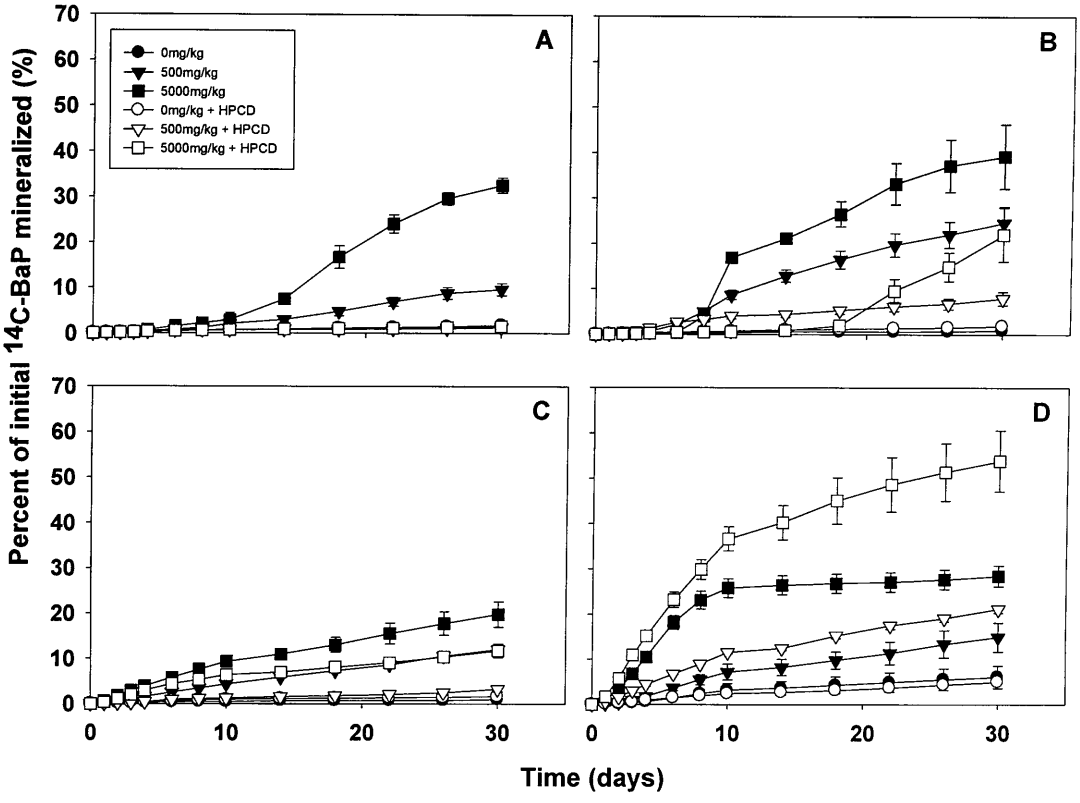


Figure 2: Mineralisation of ^{14}C-B[a]P (10 mg kg^{-1}) by indigenous microorganisms in (A) Antarctic (B) Nether-Kellet (C) Holme and (D) Thurnham soils. Legends: Unsupplemented systems: 0 (●), 500 (▼) and 5000 (■) $mg_{oil\text{-}C}$ kg^{-1}_{soil}. HP-β-CD supplemented (50 mM) systems: 0 (○), 500 (▽) and 5000 (□) $mg_{oil\text{-}C}$ kg^{-1}_{soil}. MBS+diesel systems: 500 + 500 (♦) and 5000 + 500 (◊) $mg_{oil\text{-}C}$ kg^{-1}_{soil}. The symbols represent means and the bars where visible are standard error the means (n = 3) [Adapted from Paper VII].

Paper II

Impact of diesel oil concentrations and soil contact time on naphthalene and benzo[a]pyrene mineralisation in soil

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Abstract

The impact of diesel – applied as log increments (1–10 000 $mg_{oil\text{-}C}$ kg^{-1}_{soil}) on the evolution of indigenous catabolic activities towards ^{14}C-naphthalene and ^{14}C-benzo[a]pyrene was investigated after 1, 21, 42 and 63 d soil–diesel contact time. Diesel concentration effect (C_{effect}), defined as ^{14}C-PAH mineralisation rates in amended soil normalised to rates in unamended soil, indicated that naphthalene catabolic activity was significantly enhanced along diesel concentration-gradient up to 1000 $mg_{oil\text{-}C}$ kg^{-1}_{soil}, but was progressively repressed at 10 000 $mg_{oil\text{-}C}$ kg^{-1}_{soil}. Whilst benzo[a]pyrene catabolic activity was marginally enhanced by diesel at 10–100 $mg_{oil\text{-}C}$ kg^{-1}_{soil} only after 42 d, it was immediately and significantly enhanced at 1000–10 000 $mg_{oil\text{-}C}$ kg^{-1}; being sustained for longer period at 1000 $mg_{oil\text{-}C}$ kg^{-1}_{soil}. Mineralisation data were complemented by similar temporal changes in microbial abundance, measured as CFUs for diesel- and PAH-degrading bacteria. It is suggested that rapid nutrient depletion and increased toxicity at higher diesel concentrations progressively increased the repression of PAH catabolic activity in soil.

Capsule:

Development of indigenous catabolic activity towards naphthalene and benzo[a]pyrene is enhanced or repressed in soil depending on the initial diesel concentration and soil contact time.

Keywords: *Catabolic activity; Benzo[a]pyrene; Diesel oil; Naphthalene; Soil*

Highlights:

- ▶ Impact of diesel concentrations on development of PAH catabolism was investigated.
- ▶ Increasing diesel concentration (1–1000 mg kg^{-1}) enhanced naphthalene catabolism.
- ▶ At 10,000 mg kg^{-1}, naphthalene catabolism was repressed after initial enhancement.
- ▶ Diesel (1–100 mg kg^{-1}) had negligible effect on B[a]P catabolism.
- ▶ Enhanced B[a]P catabolism was sustained longer at 1000 than at 10,000 mg kg^{-1}.

1. Introduction

Large quantities of polycyclic aromatic hydrocarbons (PAHs) are deposited in soil through natural incidents, such as wildfires and incomplete combustion of organic matter, through anthropogenic activities; for example, through exploration and transport of crude oil and refined petroleum products, as well as via deliberate discharge or accident spills [1]. PAHs are of serious environmental and health concern because they often exhibit toxic, carcinogenic and mutagenic properties [2] and their persistence due to recalcitrant aromatic structure, low aqueous solubility and high hydrophobicity, which render these molecules resistant to physical and chemical loss processes in the environment [3]. A major loss process of PAHs in soil is through microbial activity, which is strongly influenced by PAH bioaccessibility, soil physicochemical properties, such as organic matter content and inherent catabolic potential of soil microflora [4,5], as well as the composition and concentration of co-contaminants [6].

Studies on the development of indigenous PAH degradative activity in soil have focused primarily on exposure to PAHs applied as single contaminants, and as binary or multiple contaminant mixtures with fewer numbers of studies having focused on soils polluted with complex co-contaminant mixtures, such as creosote, coal tar or diesel [6-9]. So far, where such studies have been carried out, more have investigated the low-molecular-weight PAHs (LMW-PAHs) [10-12] rather than the high-molecular-weight PAHs (HMW-PAHs) [13-15], despite HMW-PAHs posing a greater risk to the environment and human health [3]. Moreover, most of the studies on HMW-PAHs have been mainly concerned with the effects of co-contaminants on bioavailability and/or biodegradation and not on the development of indigenous degradative activity. For example, Kanaly et al. [14] and Kanaly and Watanabe [15] used an enriched

bacterial consortium isolated from soil previously exposed to diesel to degrade benzo[a]pyrene dissolved in various compositions of hydrocarbon mixtures. The mechanisms and processes for the development of indigenous degradative activity especially toward HMW-PAHs in the presence of co-contaminants in soils remain poorly understood.

Further, the effects of pre-exposure to complex co-contaminant mixtures may be more complicated than have been observed in such studies with single, binary or multiple contaminant mixtures. Therefore, it is most desirable to understand what factors influence the development of indigenous catabolic activity in soils exposed to complex contaminant mixtures. The effect of co-contaminant concentration and soil contact time on the development of indigenous catabolic activity has not been fully investigated under complex contaminant mixtures systems. In particular, there is limited information in the literature on the development of indigenous catabolic activity for benzo[a]pyrene along concentration-gradient in soil polluted with complex co-contaminant mixtures. Obviously, such understanding has important implications because contaminant concentrations varied widely in contaminated sites even on a millimeter/centimeter-scale. Further, knowledge of the effects of exposure concentration and history under complex co-contaminants systems is of importance in designing and evaluating bespoke strategies for contaminated land clean up.

Therefore, this present study aimed to comparatively assess the impacts of increasing diesel concentrations and soil contact times on the development of indigenous catabolic activity towards two model PAHs in a pristine soil. Naphthalene is a "readily biodegradable" 2-ring LMW-PAH while benzo[a]pyrene is a "recalcitrant" 5-ring HMW-PAH.

2. Materials and methods

2.1. Materials

Naphthalene and benzo[a]pyrene (>99%) and [7-^{14}C]naphthalene (55 mCi mmol^{-1}, >99.6%) were purchased from Sigma–Aldrich, UK and [7-^{14}C]benzo[a]pyrene (13.8 mCi mmol^{-1}, >95%) from Amersham Corp., USA. Goldstar liquid scintillation cocktails and 7-ml glass scintillation vials were supplied by Meridian, UK, sodium hydroxide (NaOH) by Merck, UK, chemicals for the minimal basal salt (MBS) solution by Fisher Scientific, UK, and the agar powder by Oxoid Ltd, UK. Diesel oil was obtained from a BP fuel station in Lancaster, UK (specific gravity 0.85, C-content 87%; information from the supplier).

2.2. Soil amendment with diesel oil

Soil classified as typical Stagnogley (A horizon; 5–20 cm) was collected from a research field at Myerscough College (SD496402), near Lancaster, UK. The soil has a sandy loam texture, pH of 6.53 and particle size distribution: 55.63% sand, 24.96% silt, 19.41% clay, with 4.82% organic matter [16]. The soil had 29.54 μg ∑16 USEPA PAH kg^{-1}: naphthalene (1.07), fluorene (0.58), phenanthrene (17.70), and anthracene (9.03), chrysene (0.41), benzo[*b*+*k*]fluoranthene (0.63) and indeno[1,2,3-cd]pyrene (0.12). Prior to soil amendment, sieved soil samples (<2-mm) were equilibrated at 21 °C for 10 d to allow microbial activity to be optimised. Spiking of diesel to soil followed the single spiking/rehydration of air-dried soil procedure described by Doick et al. [17] to give final nominal concentrations of 0, 1, 10, 100, 1000 and 10000 $mg_{oil\text{-}C}$ kg^{-1} soil. The effectiveness of this procedure to deliver the spike concentrations was verified using gas chromatography coupled to a flame ionisation detector. The effectiveness increases from 60 to 108% with increasing diesel concentration. Moisture

120 contents of the amended soils were maintained at 60% of water holding capacity
121 (WHC) throughout the incubation, carried out in the dark at 21 ± 1 °C. At 1, 21, 42 and
122 63 d soil-diesel contact time, samples were taken and analysed as described in the
123 following sections.
124
125 *2.3. Enumeration of microbial cell numbers in soils*
126 At each contact time, culturable microbial cells in unamended and diesel-amended soils
127 were enumerated as colony forming units (CFUs) using the viable plate counts
128 techniques [18]. Diesel, naphthalene or benzo[a]pyrene (25 mg l^{-1}) served as a sole
129 carbon source for hydrocarbon-degrading microorganisms. The plates were incubated
130 at 25 °C, examined for microbial growth every other day and the CFUs counted after
131 approximately 8–10 d.
132
133 *2.4. Mineralisation of ^{14}C-PAHs in soils*
134 Measurements of ^{14}C-PAH catabolism were carried out, in triplicate, in modified 250-
135 ml Schott bottles with Teflon-lined screw-caps to which a 7-ml vial containing 1 M
136 NaOH (1 ml) to capture $^{14}CO_2$ was fitted [19]. Each respirometer containing 10 ± 0.2 g
137 of soil with 30 ml of autoclaved minimal basal salts (MBS) solution [20] was spiked
138 with $^{12}/^{14}$C-naphthalene (50 mg kg^{-1}) or $^{12}/^{14}$C-benzo[a]pyrene (10 mg kg^{-1}). The
139 respective ^{14}C-PAH activity spiked was 83 kBq kg^{-1} soil DW. A set of respirometers
140 containing unamended soil with no ^{14}C-PAHs was included for analytical corrections
141 of background radioactivity. The respirometers were incubated in the dark at 21 ± 1 °C
142 on an orbital shaker (Janke and Kunkel, IKA®-Labortechnik KS501D, Fisher
143 Scientific, UK) for continuous aeration at 100 rpm and sampled at defined intervals
144 over 18 d. The $^{14}CO_2$ trapped was mixed with 5 ml Goldstar liquid scintillation

cocktails and stored in the dark for 24 h to normalise the effects of chemo-luminescence. The ^{14}C-activity was quantified on a Tri-Carb 2300TR liquid scintillation counter (Canberra Packard, Belgium). Overall extent of mineralisation (%); fastest rate of mineralisation (% d^{-1}) and lag phase (d; time before 5% of added ^{14}C-PAH is mineralised) were calculated from the mineralisation data.

2.5. Statistical analysis

To compare the impacts of increasing diesel concentrations and soil-diesel contact times on ^{14}C-PAH mineralisation parameters, two-way analysis of variance (ANOVA) was conducted using SigmaStat statistical software version 3.5 (SPSS, USA). Where the *F*-statistic from the ANOVA showed significance difference, Tukey's Least Significance Difference was used to determine which samples differed ($P < 0.05$).

3. Results

3.1. Impact of diesel concentration and soil contact time on microbial cell numbers

At each contact time, the CFUs for diesel-, naphthalene- and benzo[a]pyrene-degrading bacteria were determined (Table 1). Generally, the CFUs of diesel-degrading microorganisms increased in all amended soils and their numbers were maintained above the background levels for longer periods in soils amended at high concentrations (1000–10 000 $mg_{oil\text{-}C}\ kg^{-1}_{soil}$) than at lower concentrations (1–100 $mg_{oil\text{-}C}\ kg^{-1}_{soil}$). Up to 21 d after soil–diesel contact, naphthalene-degrading numbers were greater in 10–100 $mg_{oil\text{-}C}\ kg^{-1}_{soil}$ than in 1000–10 000 $mg_{oil\text{-}C}\ kg^{-1}_{soil}$ treatments. However, as soil–diesel contact time increased, the numbers of naphthalene-degrading microorganisms gradually decreased in 10–100 $mg_{oil\text{-}C}\ kg^{-1}_{soil}$ treatments, but continued to increase in 1000 $mg_{oil\text{-}C}\ kg^{-1}_{soil}$ treatment. There was also a significant decline in the CFUs of

170 naphthalene degraders in 10 000 $mg_{oil\text{-}C}$ kg^{-1}_{soil} treatment by 63 d of soil–diesel contact.
171 In this study, increased soil–diesel contact time resulted in significant increases ($P <$
172 0.05) in the numbers of benzo[a]pyrene degrading microorganisms in 1000–10 000 mg
173 kg^{-1}_{soil} but not in 1–100 $mg_{oil\text{-}C}$ kg^{-1}_{soil} treatments. Of the total numbers of
174 microorganisms that grew in the presence of diesel, substantially more ($P < 0.05$) had
175 the ability to grow on naphthalene than benzo[a]pyrene as a sole source of carbon and
176 energy.
177
178 *3.2. Impact of diesel concentration and soil contact time on mineralisation of ^{14}C-*
179 *naphthalene*
180 Mineralisation of freshly added ^{14}C-naphthalene by indigenous soil microflora was
181 monitored in unamended and diesel-amended soils over time (Figure 1; Table 2).
182 Mineralisation was relatively rapid (lag phases <4 d) and high (fastest rates 9–14% d^{-1};
183 overall extents 35–45%) in the unamended soil at all sampling times. At 1 d soil–diesel
184 contact time, the lag phases, fastest rates and overall extents of mineralisation were
185 comparable ($P > 0.05$) in the 1–1000 $mg_{oil\text{-}C}$ kg^{-1}_{soil} treatments to the unamended soil.
186 However, as soil–diesel contact time increased, the lag phases became shorter, but
187 fastest rates and extents remained comparable in the 1–1000 $mg_{oil\text{-}C}$ kg^{-1}_{soil} treatments
188 and the unamended soil. Further, the times for rates to peak (T_{max}) became shorter in
189 the 1–1000 $mg_{oil\text{-}C}$ kg^{-1}_{soil} treatments (averaged 5 d at 1 d soil–diesel contact time and
190 reduced to 3 d at other times) compared to unamended soil (averaged 5 d at all times).
191 Meanwhile, the effect of the highest diesel concentration investigated (10 000 $mg_{oil\text{-}C}$
192 kg^{-1}) on different aspects of ^{14}C-naphthalene mineralisation was dependent on soil–
193 diesel contact time. In comparison to the unamended soil and other soil treatments, the
194 lag phases were significantly longer ($P < 0.05$) in the 10 000 $mg_{oil\text{-}C}$ kg^{-1}_{soil} treatment,

195 at all sampling times. Both the fastest rates and overall extents were significantly
196 higher ($P < 0.05$) at 1 d contact time, became comparable ($P > 0.05$) after 21 and 42 d
197 contact times. Unexpectedly however, mineralisation was significantly inhibited after
198 63 d of soil–diesel contact.
199
200 *3.3. Impact of increasing diesel concentration and soil contact time on mineralisation*
201 *of ^{14}C-benzo[a]pyrene*
202 The mineralisation of freshly added ^{14}C-benzo[a]pyrene by indigenous soil microflora
203 was monitored in unamended and amended soils at each sampling time (Figure 2;
204 Table 3). There was negligible ^{14}C-benzo[a]pyrene mineralisation (extents <1%) in the
205 unamended soil, at all contact times. Mineralisation was also negligible (extents <5%)
206 with extended lag phases (>18 d) in 1–100 $mg_{oil\text{-}C}\ kg^{-1}_{soil}$ treatments at all sampling
207 times. Meanwhile, ^{14}C-benzo[a]pyrene mineralisation was significantly enhanced in
208 1000–10 000 $mg_{oil\text{-}C}\ kg^{-1}_{soil}$ treatments. The lag phases were usually shorter in 1000
209 $mg_{oil\text{-}C}\ kg^{-1}_{soil}$ (11 d) than in 10 000 $mg_{oil\text{-}C}\ kg^{-1}_{soil}$ treatment (12–>18 d). For all
210 sampling times, the extents of mineralisation ranged from 12 to 14% in the 10 000
211 $mg_{oil\text{-}C}\ kg^{-1}_{soil}$ treatment; this being highest after 21 d soil–diesel contact. At 1 and 21 d
212 sampling times, the extents of mineralisation ranged from 20 to 25% in the 10 000
213 $mg_{oil\text{-}C}\ kg^{-1}_{soil}$ treatment; this being higher at 1 d soil–diesel contact. However, these
214 relatively high extents of mineralisation significantly reduced to <3% after 42 and 63 d
215 soil–diesel contact times. In the 1–100 $mg_{oil\text{-}C}\ kg^{-1}_{soil}$ treatments where mineralisation
216 extent did not exceed 5%, T_{max} was not attained before the bioassay was terminated. In
217 the 1000–10 000 $mg_{oil\text{-}C}\ kg^{-1}_{soil}$ treatments where appreciable mineralisation was
218 observed, T_{max} ranged 10–18 d, indicating the variability in catabolic potentials of
219 individual microbial population involved in the degradation.

220

221 *3.4. Use of C_{effect} to interpret the impact on evolution of PAH catabolic activity*

222 To gain further insights into the impacts of diesel oil concentrations on the evolution of
223 PAH catabolic activity, the effect of concentration (C_{effect}) was investigated. In the
224 context of this paper, evolution is used to describe the initiation and expression of
225 catabolic activity within microbial community in response to the presence of a
226 substrate. The C_{effect} is defined as the rate of PAH mineralisation in an amended soil
227 normalised to the rate in the unamended soil; an increase (>1) or a decrease (<1) in the
228 C_{effect} indicates enhancing or repressing effects, respectively. Although the extents of
229 ^{14}C-naphthalene mineralisation suggested there were no statistically significant effects
230 ($P > 0.05$), the C_{effect} graphs indicated that naphthalene catabolic activity was
231 significantly enhanced, by 2- to 8-times along a diesel concentration-gradient up to
232 1000 $mg_{oil\text{-}C}$ kg^{-1} (Figure 3). This enhancement was immediate and brief (usually lasted
233 for 4 d only); the magnitude of the enhancing effect initially increased and then
234 gradually declined as soil–diesel contact time increased. The C_{effect} graphs indicated a
235 progressive increase in the repressing effect on naphthalene catabolic activity in the 10
236 000 $mg_{oil\text{-}C}$ $kg^{-1}{}_{soil}$ treatment. Further, the C_{effect} graphs showed that benzo[a]pyrene
237 catabolic activity was enhanced, though relatively marginally, in the 10–100 $mg_{oil\text{-}C}$ $kg^{-1}{}_{soil}$
238 treatments as soil–diesel contact time increased; this being 2- to 5-times higher
239 after 42 d and increased to 5- to 7-times after 63 d soil–diesel contact (Figure 4). At
240 higher diesel concentrations (1000–10 000 $mg_{oil\text{-}C}$ kg^{-1}), graphs showed that the
241 benzo[a]pyrene catabolic activity was significantly enhanced and that the magnitude
242 was dependent on diesel concentration and soil contact time.

243

4. Discussion

Consistent with the results of this study, the soil has previously been reported to harbour bacteria possessing the degradative ability to mineralise aliphatic hydrocarbons and LMW-PAHs such as naphthalene, but not HMW-PAHs such as benzo[a]pyrene [6,9,21]. The predominance of LMW-PAHs reflects the pristine nature of the soil in that naphthalene, phenanthrene and perylene are thought to be produced biologically and are at higher levels in soils dominated by a background source pattern while the HMW-PAHs are dominant in soils heavily influenced by anthropogenic PAH input [22]. The rapid and extensive mineralisation of ^{14}C-naphthalene coupled with the large numbers of naphthalene-degrading microorganisms provides strong evidence of the presence of high potential for naphthalene catabolism in this pristine soil [6,3,9].

The ^{14}C-naphthalene mineralisation kinetics for all soil treatments at all contact times followed the typical sigmoidal curve. Similar mineralisation kinetics observed by other workers has been related to microbial growth-linked degradation of PAHs [17,13]. As soil–diesel contact time increased, the rates and extents of ^{14}C-naphthalene mineralisation remained comparable but the lag phases were shorter in the 1–1000 mg kg^{-1}_{soil} treatments than in the unamended soil. The results are comparable with the findings of Swindell and Reid [12] who reported that diesel, applied at a range of concentrations (0–2000 mg kg^{-1}_{soil}) did not affect the rates and extents of phenanthrene mineralisation in soil. However, their results differ from ours in that diesel did not also reduce the lag phase. Transformer oil applied at 100–1000 mg kg^{-1}_{soil} was showed to significantly reduced the lag phase as well as increased rates and extents of phenanthrene mineralisation after ≥28 d soil–oil contact [11].

The poor mineralisation of ^{14}C-benzo[a]pyrene in the unamended soil is consistent with previous reports on this soil [9,24] and other soils [25,13]. Low benzo[a]pyrene

catabolic activity in soil can be linked, in part, to the compound's physicochemical characteristics – low water solubility (0.0038 mg l^{-1}) and high octanol-water partition coefficient (log K_{ow} 6.06), as well as the highly-recalcitrant chemical structure and the inability to readily support microbial growth [26] and, in part, to low populations of benzo[a]pyrene degraders in this soil. In addition, low levels of benzo[a]pyrene from natural sources in soil and poor distribution or lack of endogenous benzo[a]pyrene degradation traits in the gene pools within microbial communities can limit adaptation and development of degradative ability by most soil microflora [27].

The results of ^{14}C-benzo[a]pyrene mineralisation in the amended soils indicated that a long period of adaptation was required for the development of benzo[a]pyrene catabolic activity in soil amended at low diesel concentrations (1–100 mg kg^{-1}). This is attributable to slow growth rates of the arrays of microbial species necessary to complete the degradation process and to the inadequacy of diesel to serve as co-solvent as well as to supply sufficient co-substrates at low concentrations [15]. As observed in this study, the trend by which the lag phases were relatively shorter in the 1000 mg kg^{-1}_{soil} compared to the 10 000 mg kg^{-1}_{soil} treatment has previously been reported. The longest lag phases were observed at the highest diesel fuel concentration when a bacterial consortium was used to mineralise ^{14}C-benzo[a]pyrene in liquid medium [28] and in soil [29]. This was attributed to the preferential metabolism of certain components of diesel, which resulted in an initial competitive inhibition of enzymes associated with benzo[a]pyrene degradation.

In soil treatments wherein ^{14}C-benzo[a]pyrene mineralisation was substantial, the kinetics showed atypical growth curves with extended lag phases, which often describe the mineralisation of refractory chemicals that hardly support fast microbial proliferation [30]. This kind of ^{14}C-benzo[a]pyrene mineralisation is consistent with the

294 findings of other investigators [15]. At sufficient concentrations, certain components of
295 diesel, such as high-boiling point distillate fractions, supported extensive
296 mineralisation of benzo[a]pyrene by acting as co-solvent for PAH dissolution and/or as
297 inducers of co-metabolism as well as promoting the growth of catabolically-competent
298 microbial populations in soil [15]. In this study, there is no conclusive evidence to
299 confirm whether ^{14}C-benzo[a]pyrene mineralisation proceeded via cometabolic
300 degradation; however, it is clear from the results that there was a concentration-
301 dependence of diesel to support benzo[a]pyrene-degrading microflora and enhanced
302 benzo[a]pyrene mineralisation in soil.
303 The C_{effect} graphs provided a better illustrative description of the effect of diesel
304 concentration on the initiation and expression of PAH catabolic activity than the
305 conventional indices of mineralisation and explained why the overall extents of
306 mineralisation were not significantly different in the unamended and amended soils at
307 the end of the 18-d bioassay. Firstly, the evolution of naphthalene catabolic activity in
308 the 1–1000 $mg_{oil\text{-}C}\ kg^{-1}{}_{soil}$ treatments was immediate and higher than in the unamended
309 soil (i.e. the C_{effect} values immediately increased to >1). Secondly, the faster removal of
310 the bioavailable fraction of the added ^{14}C-naphthalene in these amended soils caused
311 the initially enhanced catabolic activity to regress to levels in the unamended soil (i.e.
312 the C_{effect} values rapidly dropped to *ca.*1). This trend was also reflected by the relatively
313 shorter T_{max} observed in the 1–1000 $mg_{oil\text{-}C}\ kg^{-1}{}_{soil}$ treatments. In a soil with high
314 degradative ability for phenanthrene, Semple et al. [31] demonstrated that the
315 termination of mineralisation was due to the removal of the bioaccessible fraction of
316 the chemical and not decreasing cellular activity or cell death. In the case of
317 benzo[a]pyrene, although the initiation was delayed, expression of catabolic activity

318 was significantly higher (>1) in the 1000–10 000 $mg_{oil\text{-}C}\ kg^{-1}{}_{soil}$ treatments than in the
319 unamended soil.
320 Previously, it was reported that diesel, at concentrations of 0–2000 $mg_{oil\text{-}C}\ kg^{-1}{}_{soil}$, did
321 not enhance indigenous catabolic activity, based on the comparable values of the
322 overall extents of ^{14}C-phenanthrene mineralisation in the unamended and amended
323 soils [12; Figure 4]. However, it is suggested that were their data interpreted using the
324 C_{effect} it would be seen that pre-exposure to diesel at these concentrations actually
325 enhances the catabolic activity of the indigenous soil microflora, but that chemical
326 bioavailability has a profound influence on the extent of PAH mineralisation.
327 Therefore, interpreting mineralisation data using the C_{effect} can provide additional
328 insights to the spatial and temporal localisation of catabolic activities in soils
329 contaminated with complex co-contaminant mixtures along concentration-gradients.
330 This has implication in sustaining and/or enhancing catabolic activity by improving
331 PAH bioavailability during bioremediation of contaminated soil.
332 In the 10 000 mg $kg^{-1}{}_{soil}$ treatment, the C_{effect} data revealed a progressive increase in the
333 repression of indigenous catabolic activity which was indiscriminate toward both
334 naphthalene and benzo[a]pyrene, despite the obvious differences in the
335 physicochemical characteristics and amenability of the PAHs to microbial degradation.
336 This phenomenon can be attributed to the rapid depletion of essential nutrients (e.g.,
337 nitrogen and phosphorus) and oxygen in soil and/or the increased toxicity at higher
338 diesel concentrations. As observed particularly in the 1000–10 000 $mg_{oil\text{-}C}\ kg^{-1}{}_{soil}$
339 treatments, rapid increase in the populations of hydrocarbon-degrading bacteria may
340 result in considerable depletion of requisite inorganic nutrients (e.g., nitrogen and
341 phosphorus) and oxygen in soil [10,32]. A number of studies has also reported the
342 repression of PAH catabolic activity of indigenous soil microorganisms as a result of

343 the toxic nature of diesel at higher concentrations and/or to increased toxicity due to
344 accumulation of dead-end metabolites [6, 33]. In a study of the influence of chemical
345 structure, concentration and multiple amendment on biodegradation of PAHs in soil, a
346 decrease to the extent of ^{14}C-naphthalene mineralisation in soil spiked with the PAHs
347 phenanthrene and pyrene applied at 75–300 mg kg^{-1} was recorded after ≥56 d soil–
348 PAH contact time [6]. The authors suggested that accumulation of toxic metabolic
349 intermediates or the general toxic properties of the PAHs caused the decline in the
350 degrading microbial numbers and their catabolic activity.
351 In conclusion, the results collectively emphasise that the enhancement or repression of
352 indigenous catabolic activity towards naphthalene and benzo[a]pyrene in soil is
353 dependent on initial exposure concentration and residence time of diesel in soil.
354

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358

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455 **Figures**

456 Figure 1: Impact of increasing diesel concentration and contact time on ^{14}C-
457 naphthalene mineralisation in soil amended with diesel at 0 mg kg^{-1} (•), 1 mg kg^{-1} (○),
458 10 mg kg^{-1} (▼), 100 mg kg^{-1} (Δ), 1000 mg kg^{-1} (■) and 10 000 mg kg^{-1} (□) after 1 d
459 (A), 21 d (B), 42 d (C), and 63 d (D) soil–diesel contact times.

460 Figure 2: Impact of increasing diesel concentration and contact time on ^{14}C-
461 benzo[a]pyrene mineralisation in soil amended with diesel at 0 mg kg^{-1} (•), 1 mg kg^{-1}
462 (○), 10 mg kg^{-1} (▼), 100 mg kg^{-1} (Δ), 1000 mg kg^{-1} (■) and 10 000 mg kg^{-1} (□) after 1
463 d (A), 21 d (B), 42 d (C), and 63 d (D) soil–diesel contact times.

464 Figure 3: Concentration effect (C_{effect}) on the evolution of naphthalene catabolic
465 activity in soil amended with diesel at 0 mg kg^{-1} (•), 1 mg kg^{-1} (○), 10 mg kg^{-1} (▼),
466 100 mg kg^{-1} (Δ), 1000 mg kg^{-1} (■) and 10 000 mg kg^{-1} (□) after 1 d (A), 21 d (B), 42 d
467 (C), and 63 d (D) soil–diesel contact times.

468 Figure 4: Concentration effect (C_{effect}) on the evolution of benzo[a]pyrene catabolic
469 activity in soil amended with diesel at 0 mg kg^{-1} (•), 1 mg kg^{-1} (○), 10 mg kg^{-1} (▼),
470 100 mg kg^{-1} (Δ), 1000 mg kg^{-1} (■) and 10 000 mg kg^{-1} (□) after 1 d (A), 21 d (B), 42 d
471 (C), and 63 d (D) soil–diesel contact times.

472 **Tables**

473 Table 1: Impacts of increasing diesel concentration and soil contact time on indigenous
474 degrader numbers (CFU g^{-1}). Values in parentheses are standard error of mean (n = 3).

475 Table 2: Impacts of increasing diesel concentration and soil contact time on indigenous
476 microbial mineralisation of ^{14}C-naphthalene.

477 Table 3: Impacts of increasing diesel concentration and soil contact time on indigenous
478 microbial mineralisation of ^{14}C-benzo[a]pyrene.

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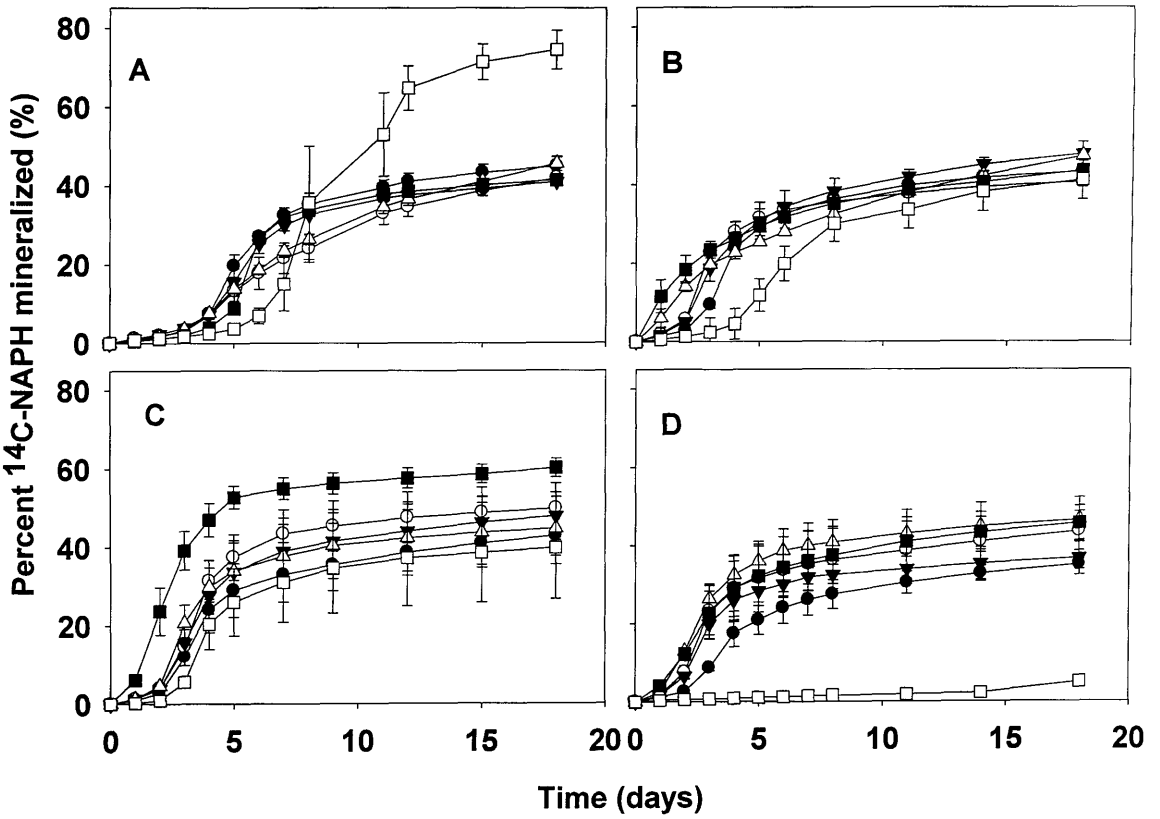

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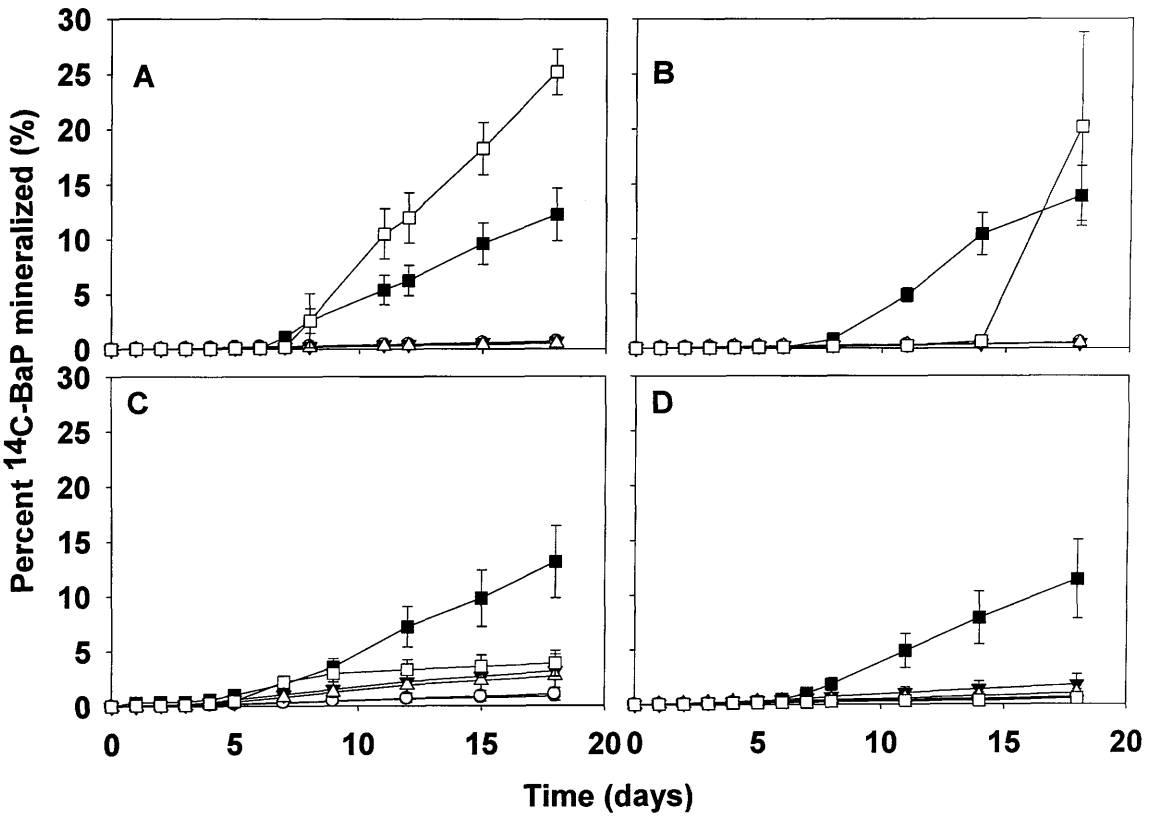

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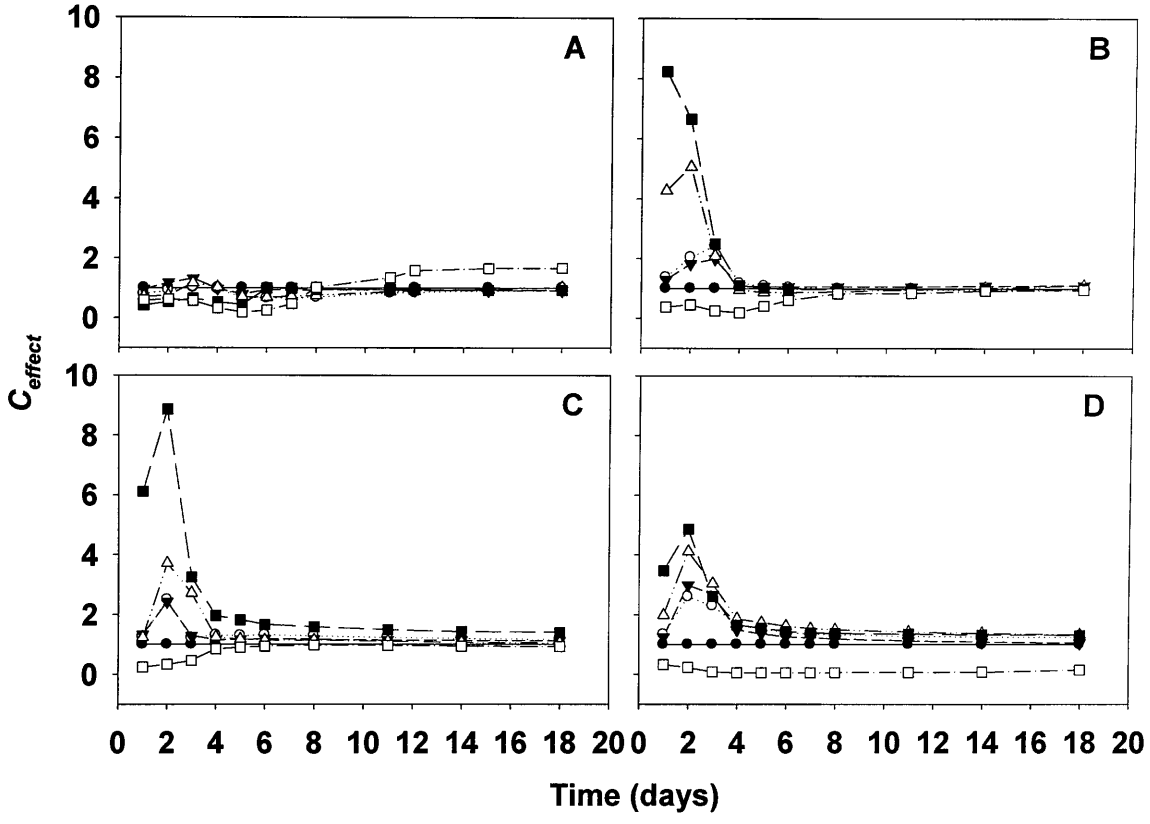

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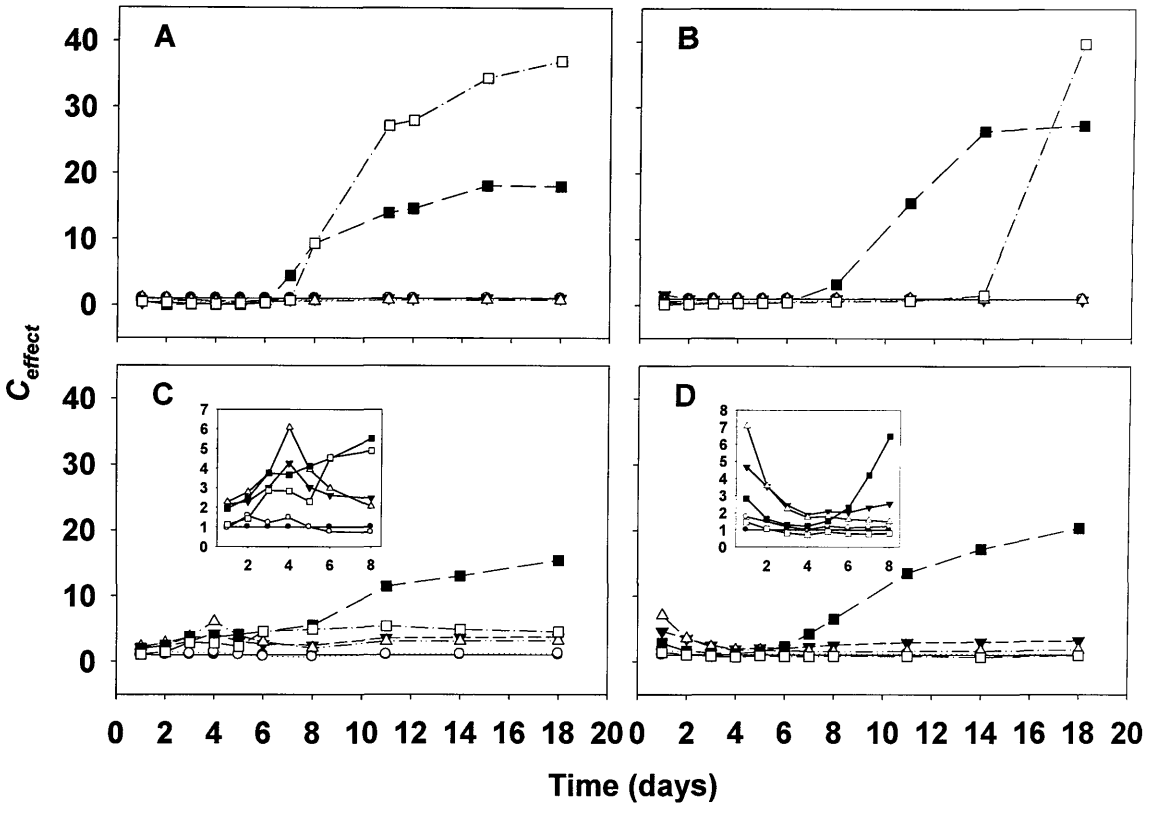

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Time (d)	Concentration ($mg_{oil\text{-}C}\ kg^{-1}_{soil}$)	Degrader numbers (x 10^6 CFUs g^{-1})		
		Diesel	Naphthalene	Benzo[a]pyrene
1	0	2.17 (0.33)	2.08 (1.66)	0.01 (0.01)
	1	2.20 (0.42)	2.13 (1.03)	0.01 (0.04)
	10	47.30 (4.98)	38.70 (5.88)	0.11 (0.02)
	100	33.00 (1.53)	30.30 (2.03)	0.13 (0.01)
	1000	17.30 (4.67)	20.80 (1.36)	0.97 (0.09)
	10 000	18.00 (1.16)	14.90 (3.64)	0.70 (0.06)
21	0	2.67 (0.88)	2.40 (0.32)	0.02 (0.01)
	1	2.33 (0.33)	2.20 (0.38)	0.01 (0.00)
	10	53.30 (0.67)	44.00 (0.26)	0.03 (0.01)
	100	60.30 (5.80)	60.20 (0.17)	0.40 (0.02)
	1000	79.40 (8.08)	48.30 (7.30)	1.00 (0.03)
	10 000	39.80 (5.80)	30.90 (3.90)	1.00 (0.08)
42	0	3.33 (0.33)	2.23 (0.79)	0.01 (0.00)
	1	2.67 (0.88)	2.27 (0.82)	0.02 (0.01)
	10	24.33 (1.20)	17.20 (0.50)	0.05 (0.01)
	100	36.37 (1.92)	17.50 (0.56)	0.04 (0.00)
	1000	263.0 (81.0)	68.30 (7.40)	0.99 (0.24)
	10 000	33.30 (1.20)	27.30 (10.10)	0.63 (0.08)
63	0	2.17 (0.24)	2.63 (0.76)	0.03 (0.01)
	1	2.75 (0.33)	3.83 (0.07)	0.03 (0.00)
	10	15.00 (1.53)	13.37 (0.58)	0.04 (0.00)
	100	27.67 (2.60)	15.87 (0.43)	0.03 (0.01)
	1000	70.80 (5.00)	45.30 (1.32)	0.59 (0.04)
	10 000	5.10 (2.52)	9.30 (0.49)	0.29 (0.24)

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Time (d)	Concentration ($mg_{oil\text{-}C}\ kg^{-1}_{soil}$)	Lag time (d)	Fastest rate (% d^{-1}); T_{max} (d)	Overall extent (%)
1	0	3.55 ± 0.20 ^{aA§}	12.07 ± 1.60 ^{aA} (5–6)‡	44.89 ± 2.25 ^{aA}
	1	3.52 ± 0.24 ^{aA}	10.20 ± 1.83 ^{aA} (5)	41.83 ± 0.75 ^{aA}
	10	3.44 ± 0.26 ^{aA}	9.68 ± 1.47 ^{aA} (4–5)	40.91 ± 0.89 ^{aA}
	100	3.38 ± 1.26 ^{aA}	10.25 ± 0.25 ^{aA} (4–5)	45.54 ± 1.85 ^{aA}
	1000	4.89 ± 0.00 ^{aA}	10.97 ± 0.39 ^{aA} (6)	41.37 ± 1.43 ^{aA}
	10 000	5.53 ± 0.36 ^{aC}	20.68 ± 4.18 ^{bA} (6–8)	74.39 ± 4.91 ^{bA}
21	0	2.35 ± 0.05 ^{aB}	14.42 ± 2.35 ^{aA} (4)	43.30 ± 3.15 ^{aA}
	1	1.83 ± 0.11 ^{aB}	17.13 ± 2.29 ^{aB} (3)	40.68 ± 1.39 ^{aA}
	10	1.91 ± 0.13 ^{aB}	13.64 ± 3.88 ^{aB} (3)	47.74 ± 2.98 ^{aA}
	100	0.91 ± 0.22 ^{bB}	17.99 ± 1.37 ^{aB} (2)	47.15 ± 0.61 ^{aA}
	1000	0.60 ± 0.26 ^{bB}	11.57 ± 4.19 ^{aA} (1–2)	43.51 ± 2.29 ^{aA}
	10 000	4.07 ± 0.01 ^{cB}	11.06 ± 3.24 ^{aB} (6–8)	41.13 ± 5.07 ^{aB}
42	0	2.27 ± 0.06 ^{aB}	11.96 ± 6.03 ^{aA} (4–5)	42.91 ± 5.23 ^{aA}
	1	2.10 ± 0.02 ^{abB}	16.73 ± 3.39 ^{aB} (4)	49.84 ± 6.44 ^{aA}
	10	2.13 ± 0.06 ^{abB}	12.05 ± 4.20 ^{aB} (3–4)	47.86 ± 6.12 ^{aA}
	100	1.99 ± 0.11 ^{bB}	16.11 ± 3.98 ^{aB} (3)	44.70 ± 9.18 ^{aA}
	1000	0.84 ± 0.09 ^{bB}	17.60 ± 5.96 ^{aA} (2–3)	60.22 ± 2.93 ^{aB}
	10 000	2.94 ± 0.21 ^{cA}	14.80 ± 2.53 ^{aB} (4)	39.77 ± 13.03 ^{aB}
63	0	2.41 ± 0.05 ^{aB}	8.81 ± 2.83 ^{aA} (4–5)	35.11 ± 1.25 ^{aA}
	1	1.67 ± 0.22 ^{abB}	15.64 ± 5.66 ^{aB} (3–4)	43.61 ± 5.06 ^{aA}
	10	1.71 ± 0.09 ^{abB}	13.35 ± 3.42 ^{aB} (3–4)	36.73 ± 4.36 ^{aA}
	100	1.25 ± 0.01 ^{bB}	13.48 ± 3.88 ^{aB} (2–3)	46.51 ± 5.73 ^{aA}
	1000	1.12 ± 0.04 ^{bB}	10.48 ± 3.94 ^{aA} (2–3)	45.68 ± 4.37 ^{aA}
	10 000	17.22 ± 0.41 ^{cD}	0.71 ± 0.40 ^{bC} (>18)	5.06 ± 1.08 ^{bC}

507 § Different lower-case letters down the column within each sub-group of "Time" indicate mean values of triplicate
508 samples that are significantly different ($P < 0.05$). Different upper-case letters down the column for corresponding
509 "Concentration" across groups of "Time" indicate mean values of triplicate samples that are significantly different
510 ($P < 0.05$).
511 ‡ Values in parentheses indicate range of time (d) when rates peaked in triplicates samples
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Time (d)	Concentration ($mg_{oil\text{-}C}\ kg^{-1}_{soil}$)	Lag time (d)	Fastest rate (% d^{-1}); T_{max} (d)	Overall extent (%)
1	0	>18 ^{aA}	0.06 ± 0.06 ^{aA} (>18) ‡	0.68 ± 0.33 ^{aA}§
	1	>18 ^{aA}	0.06 ± 0.02 ^{aA} (>18)	0.63 ± 0.25 ^{aA}
	10	>18 ^{aA}	0.06 ± 0.03 ^{aA} (>18)	0.70 ± 0.18 ^{aA}
	100	>18 ^{aA}	0.04 ± 0.02 ^{aA} (>18)	0.50 ± 0.21 ^{aA}
	1000	10.91 ± 1.61 ^{bA}	1.49 ± 0.59 ^{bA} (15–18)	12.26 ± 2.40 ^{bA}
	10 000	11.86 ± 2.20 ^{bA}	2.63 ± 1.95 ^{bA} (10–15)	25.20 ± 11.07 ^{bA}
21	0	>18 ^{aA}	0.06 ± 0.03 ^{aA} (>18)	0.51 ± 0.19 ^{aA}
	1	>18 ^{aA}	0.04 ± 0.01 ^{aA} (>18)	0.49 ± 0.10 ^{aA}
	10	>18 ^{aA}	0.06 ± 0.02 ^{aA} (>18)	0.45 ± 0.11 ^{aA}
	100	>18 ^{aA}	0.07 ± 0.01 ^{aA} (>18)	0.48 ± 0.15 ^{aA}
	1000	11.25 ± 0.57 ^{cA}	1.86 ± 0.49 ^{bA} (11–14)	13.90 ± 2.73 ^{bA}
	10 000	15.17 ± 0.53 ^{bA}	4.85 ± 2.06 ^{bA} (14–18)	20.20 ± 8.59 ^{bA}
42	0	>18 ^{aA}	0.11 ± 0.04 ^{aA} (>18)	0.85 ± 0.39 ^{aA}
	1	>18 ^{aA}	0.08 ± 0.01 ^{aA} (>18)	1.05 ± 0.19 ^{aA}
	10	14.22$^{\#}$ ^{bB}	0.24 ± 0.10 ^{aA} (>14)	3.16 ± 1.55 ^{aA}
	100	>18 ^{aA}	0.23 ± 0.03 ^{aB} (9–12)	2.69 ± 0.04 ^{aA}
	1000	11.31 ± 1.78 ^{bA}	1.23 ± 0.35 ^{bA} (12–18)	13.13 ± 3.28 ^{bA}
	10 000	14.40$^{\#}$ ^{bA}	0.88 ± 0.20 ^{abA}(>14)	3.86 ± 1.17 ^{aB}
63	0	>18 ^{aA}	0.05 ± 0.00 ^{aA} (>18)	0.56 ± 0.13 ^{aA}
	1	>18 ^{aA}	0.08 ± 0.01 ^{aA} (>18)	0.64 ± 0.07 ^{aA}
	10	>18 ^{aA}	0.17 ± 0.06 ^{aA} (>18)	1.79 ± 0.95 ^{aA}
	100	>18 ^{aA}	0.07 ± 0.01 ^{aA} (>18)	1.02 ± 0.35 ^{aA}
	1000	11.98 ± 1.52 ^{bA}	1.02 ± 0.32 ^{bA} (12–18)	11.42 ± 3.60 ^{bA}
	10 000	>18 ^{aB}	0.05 ± 0.05 ^{aA} (>18)	0.52 ± 0.25 ^{aB}

515 § Different lower-case letters down the column within each sub-group of "Time" indicate mean values of triplicate
516 samples that are significantly different ($P < 0.05$). Different upper-case letters down the column for corresponding
517 "Concentration" across groups of "Time" indicate mean values of triplicate samples that are significantly different
518 ($P < 0.05$).
519 ‡ Values in parentheses indicate range of time (d) when rates peaked in triplicates samples. Where value is > 18 d,
520 mineralisation did not exceed 5%.
521 # Only one of the triplicate samples reached >5% mineralisation before the end of the experiment.
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Paper III

The effects of diesel concentration and soil contact time on the development of indigenous catabolic activities toward hydrocarbons

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13 **Abstract**

14 The effect of diesel oil (applied at a log concentration range from 1 to 10,000 mg kg^{-1})
15 on the development of indigenous catabolic activity towards hydrocarbons was
16 investigated after 1, 21, 42 and 63 d soil-diesel contact times. Results indicated that the
17 evolution of ^{14}C-phenanthrene catabolic activity was significantly enhanced ($P < 0.05$)
18 for the first 42 d of soil–diesel contact, being higher at concentrations of 10–100 mg
19 kg^{-1} than at 1000 mg kg^{-1}; but was persistently repressed ($P < 0.05$) at 10,000 mg kg^{-1}.
20 Hexadecane catabolic activity in the amended soils was comparable ($P > 0.05$) to the
21 unamended soil, being marginally repressed along diesel concentration-gradient for the
22 first 21 d and minimally enhanced thereafter. The potential variability in the
23 development of indigenous catabolic activity due to the spatial heterogeneity in
24 contaminant levels should be taken into account when assessing natural attenuation of
25 contaminated sites or designing bespoke strategies for enhanced bioremediation.

26

27

28 **Capsule**:

29 The initial diesel concentration and soil contact time affect the development of
30 indigenous catabolic activity towards phenanthrene but not hexadecane.

31

32 **Keywords**: *Catabolic activity; Concentration effect; Diesel oil; n-Hexadecane;*
33 *Phenanthrene*

34

Highlights:

- *Effect of diesel concentration on hydrocarbon catabolic activity was examined*
- *Phenanthrene catabolic activity was significantly enhanced at 10–1000 mg kg^{-1}*
- *Phenanthrene catabolic activity was significantly retarded at 10,000 mg kg^{-1}*
- *Hexadecane catabolic activity was minimally affected ($P > 0.05$) at 1–10,000 mg kg^{-1}*

41 **1. Introduction**

42 Many sites contaminated with crude or refined petroleum oils are characterised by the
43 presence of complex mixtures of hydrophobic organic compounds (HOCs) which are
44 mainly aliphatic and polycyclic aromatic hydrocarbons (PAHs). For example, at
45 disused manufactured gas plants (MGPs) or sites contaminated with oil spills, high
46 levels of HOCs have been found, often contained in non-aqueous phase liquids
47 (NAPLs) (Ghoshal and Luthy, 1996; Kanaly and Harayama, 2000; Kose et al., 2003).
48 Biodegradation is the main mechanism for hydrocarbon detoxification and/or complete
49 destruction, although physical and chemical processes, such as dispersion, dilution,
50 sorption, volatilisation, and abiotic transformation, are also involved (Margesin and
51 Schinner, 2001). Bioremediation, *in situ* or *ex situ*, generally involves the
52 manipulations of microbial degradative properties and/or their environment to speed up
53 the rate of removal of hydrocarbons from soils and sediments (Mueller et al., 1997;
54 Megharaj et al., 2011).

55 In the last half century, there has been increased interest, at both research and
56 regulatory levels, to develop bespoke bioremediation technologies that explore the
57 inherent degradative properties within microbial communities to remediate
58 hydrocarbon-contaminated soils (Cerniglia, 1984; U.S. Environmental Protection
59 Agency, 1999; Juhasz et al., 2005; Serrano et al., 2008; Juhasz et al., 2010; Tang et al.,
60 2012). Meanwhile, for the successful deployment to a specific contaminated field site
61 and for a bioremediation method to be adjudged as the "best alternative technology not
62 entailing excessive costs" (BATNEEC) or as "best practicable environmental option"
63 (BPEO), there is need for a comprehensive understanding of the variety of factors that
64 may influence the development of indigenous catabolic activity in the site. The factors
65 which broadly can be categorised into environmental and edaphic, (micro)biological,

66 chemical and physical variables are known to vary widely both temporally and
67 spatially at a particular site and their combined effects may differ substantially from
68 site to site. Evidence from a series of reviews of studies showed that the complex
69 reciprocal interactions involving these factors may have significant influence on the
70 outcomes of field deployment of bioremediation technologies with mixed stories of
71 successes and failures (Bamforth and Singleton, 2005; Das and Chandran, 2011). The
72 presence of catabolically competent microorganisms is fundamental to every
73 bioremediation programme; collectively, the manipulation of these factors must be
74 geared towards ensuring the establishment and maintenance of conditions that favour
75 the development of high catabolic activity within the HOC-degrading populations.
76 There is a substantial pool of research on the effects of hydrocarbon type and
77 concentration on the development of microbial catabolic activity in soil; however, most
78 of the studies were based on single, and to lesser extents, on binary and ternary
79 hydrocarbon mixtures (Bauer and Capone, 1988; Hatzinger and Alexander, 1995;
80 Morrison and Alexander, 1997; Macleod and Semple, 2002; Stroud et al., 2007a;
81 Couling et al., 2010). The development of indigenous catabolic activity under complex
82 hydrocarbon mixture systems remained comparatively under-investigated and poorly
83 understood, though such studies are ideally more environmentally-relevant. In
84 particular, there is limited information in the literature to compare the effects of
85 contaminant concentration and soil contact time on the development of catabolic
86 activity towards the main hydrocarbon classes – *n*-alkanes and PAHs in petroleum oil-
87 contaminated soils (Swindell and Reid, 2007). Further, studies of the effect of co-
88 contaminant on the development of *n*-alkane catabolism are rare in the literature
89 despite that aliphatic hydrocarbons constitute over 90% of most refined petroleum oils

90 and make up substantial portions of the organic contaminants found as NAPLs in
91 terrestrial environments (Stroud et al., 2007b; Stroud et al., 2007a).
92 Therefore, the objective of this study was to determine and compare the effects of
93 increasing diesel concentration and soil contact time on the development of indigenous
94 catabolic activity towards a model *n*-alkane – hexadecane and a model PAH –
95 phenanthrene in a pristine soil.
96

97 **2. Materials and methods**

98 *2.1. Chemicals*

99 Phenanthrene (>96%), *n*-hexadecane (>99%), [9-^{14}C]phenanthrene (50 mCi·mmol^{-1},
100 >95%) and [1-^{14}C]*n*-hexadecane (52 mCi·mmol^{-1}, >99%) were purchased from Sigma–
101 Aldrich, UK. Goldstar liquid scintillation cocktails were obtained from Meridian, UK,
102 and sodium hydroxide from Merck, UK. Diesel oil was acquired from a BP fuel station
103 in Lancaster, UK (specific gravity 0.85, C-content 87%; information from supplier).
104 Chemicals for the minimal basal salt (MBS) solution were obtained from Fisher
105 Scientific, UK, while the agar powders used for microbiological enumeration were
106 from Oxoid Ltd., UK.
107

108 *2.2. Soil amendment with diesel oil*

109 A pristine Dystric Cambisol (sandy-loam, pH 6.53, organic matter 4.82%, sand
110 55.63%, silt 24.96%, clay 19.41%), was collected (depth 5–20 cm) from an agricultural
111 field at Myerscough College (grid reference SD496402), near Lancaster, UK. There is
112 no known history of petroleum hydrocarbon contamination; the soil had 30 μg ∑16
113 USEPA PAHs kg^{-1} consisting mainly of naphthalene (1), anthracene (9) and
114 phenanthrene (18) as determined by gas chromatography coupled to a flame ionization

115 detector (GC-FID) analysis. Hexadecane was not detectable but soil contained low
116 concentrations of identifiable nC$_{18}$–nC$_{29}$ ranged aliphatic hydrocarbons (~700 μg kg^{-1}).
117 Prior to the start of experiments, the soil was air-dried to *ca.* 40% of its water holding
118 capacity (WHC), sieved (≤2 mm) to eliminate stones, debris and plant roots and then
119 stored at 4 °C. Before diesel amendment, the soil was equilibrated at 21 °C for 10 d to
120 allow microbial activity to be optimized. Following the single-step spiking/rehydration
121 procedure described by Doick et al. (2003), soil subsamples were amended with diesel
122 to concentrations of 0, 1, 10, 100, 1000 and 10,000 mg kg^{-1}. The effectiveness (~80%)
123 of this procedure to deliver the spike concentrations was verified using GC-FID. The
124 amended soils (50 g) were placed in pre-cleaned amber glass jars (total per treatment =
125 10), loosely sealed with perforated aluminium foil and then aged in darkness under
126 controlled conditions (21 ± 1 °C, 45% relative humidity). After 1, 21, 42 and 63 d
127 contact times, subsamples were taken and analysed as described in the following
128 sections.
129
130 *2.3. Enumeration of microbial cell numbers in soil*
131 At each sampling time, bacterial cells were enumerated as colony forming units
132 (CFUs) using standard aseptic plate count techniques (Lorch et al., 1995). Briefly, soil
133 sub-sample (2 g) was placed in ¼ strength Ringer's solution (18 ml) and sonicated for
134 20 min at 100 rpm. Aliquot of soil suspension (1 ml) was then serially tenfold diluted,
135 and 0.1 ml portions of appropriate dilutions inoculated on agar plates impregnated with
136 phenanthrene or hexadecane (25 mg l^{-1}) as a sole carbon source. Plates were incubated
137 at 25 °C, and colonies counted after 8–10 d, depending on rapidity of growth.
138

139 *2.4. Mineralisation of freshly added ^{14}C-hydrocarbons in amended soils*

140 Evolution of indigenous catabolic activity was measured in amended soils using
141 respirometric assays carried out, in triplicate, in modified 250-ml Schott bottles (Reid
142 et al., 2001). Each respirometer contained the following: soil (10 ± 0.2 g), sterile MBS
143 solution (30 ml) and either ^{12}C/^{14}C-phenanthrene or *n*-hexadecane (specific density
144 0.77) at 50 mg kg^{-1} with an associated ^{14}C-activity of 83 kBq kg^{-1}. The respirometers
145 were continuously aerated (100 rpm) in the dark at 21± 1 °C and sampled at defined
146 intervals over 18 d. The $^{14}CO_2$ trapped in 1 M NaOH was mixed with 5 ml Goldstar
147 liquid scintillation cocktails and the ^{14}C-activity was quantified on Tri-Carb 2300TR
148 liquid scintillation analyser (Canberra Packard, Belgium). Cumulative extent (%),
149 fastest rate (% d^{-1}), the variability in the times taken for rates to peak, (T_{max}) and lag
150 phase (time in days prior to >5% mineralisation) of mineralisation were calculated
151 from the respirometry data.
152
153 *2.5. Modelling mineralisation kinetics*
154 Preliminary assessment of the candidate models with different hypotheses (Brunner
155 and Focht, 1984) indicated that a model with uptake and mineralisation of hydrocarbon
156 in the soil solution and sorbed phases will adequately fit the data. The choice of the
157 "best" model from the pool of suitable candidate models was based on a battery of
158 analyses (including the *F*-test method, r^2 values and residual sum of squares error,
159 RSSE) performed by nonlinear regression GLM program of SigmaStat version 3.5
160 (SPSS Software Inc., Chicago, IL, USA). A modified Gompertz model which
161 described two-compartment, three-parameter biphasic mineralisation kinetics was
162 selected and expressed mathematically as:
163 $A = A_0 \cdot (exp \cdot (-exp \cdot [-(t - t_0) / k_{aq}])) + k_{ss} \cdot t$,

where A is the extent of ^{14}C-hydrocarbon mineralisation to $^{14}CO_2$ (%) as a function of time, t; A_0 is the asymptotic yield of $^{14}CO_2$ evolved in the aqueous phase (%); k_{aq} is the first-order rate constant (d^{-1}) of the mineralisation in soil solution; t_0 is the time (d) prior to inflection point; and k_{ss} is rate constant (d^{-1}) in sorbed phase, respectively (Soulas, 1993; Morel-Chevillet et al., 1996).

2.6. Statistical analysis

Analyses of variance (ANOVA) followed by Holm-Sidak test, where necessary, was used to evaluate the effects of diesel concentration and soil contact time on ^{14}C-phenanthrene or ^{14}C-hexadecane mineralisation using SigmaStat statistical software version 3.5 (SPSS Inc., USA); significance was set at $P < 0.05$.

3. Results

3.1. Effect of diesel concentration and soil contact time on bacterial numbers

Compared to the unamended and 1 mg kg^{-1} treatment, the CFUs of phenanthrene-degrading bacteria increased considerably in the other amended soils, and were sustained for the 63-d duration of incubation in the 1000–10,000 mg kg^{-1} treatments (Table 1). The CFUs of hexadecane-degrading bacteria, though generally higher than those of phenanthrene-degrading bacteria, were unaffected by diesel concentration and contact time in soil.

3.2. Effect of diesel concentration and soil contact time on ^{14}C-phenanthrene mineralisation

The indigenous microorganisms demonstrated inherently high ability to mineralise phenanthrene; >52% of added ^{14}C-phenanthrene mineralised to $^{14}CO_2$ during the

course of an 18-d bioassay in the unamended soil, at any of the sampling times (Table 2 and Figure S1). After 1 d soil–diesel contact time, whilst the lag phases were unaffected ($P > 0.05$), fastest rates and extents of ^{14}C-phenanthrene mineralisation were increased in the 10–100 mg kg^{-1} treatments. In the 1000 mg kg^{-1} treatment, fastest rate and extent of ^{14}C-phenanthrene mineralisation were significantly ($P < 0.05$) increased although preceded by an equally significantly longer ($P < 0.05$) lag phase. After 21 d soil–diesel contact time, the lag phases were significantly shorter ($P < 0.05$) in the 1–1000 mg kg^{-1} treatments, the fastest rates were significantly higher ($P < 0.05$) in the 10–100 mg kg^{-1} treatments and the extent of mineralisation was significantly greater ($P < 0.05$) in the 10 mg kg^{-1} treatment, as compared to the unamended soil. After 42 and 63 d soil–diesel contact times, fastest rates (including T_{max}) and extents of mineralisation did not increase further ($P > 0.05$); though the lag phases remained significantly shorter ($P < 0.05$) in the 1–100 mg kg^{-1} treatments relative to the unamended soil. At all soil–diesel contact times, the lag phases were significantly longer ($P < 0.05$) while fastest rates and extents of mineralisation were significantly lower ($P < 0.05$) in the 10,000 mg kg^{-1} treatment relative to the unamended and other amended soils. However, these indices were substantially enhanced ($P < 0.05$) after 21 d and other contact times compared to at 1 d soil–diesel contact time with respect to the 10,000 mg kg^{-1} treatment. Generally, the variability in the times taken for rates to peak, (T_{max}) increased with increasing diesel concentration in soil, at any contact time.

The results of the modelled kinetic parameters for ^{14}C-phenanthrene mineralisation are presented in Table 4. A significant portion of the added ^{14}C-phenanthrene mineralised to $^{14}CO_2$ was in the aqueous phase (A_0): averaged 79% in the unamended soil and ranged from 78 to 88% in the 1–10,000 mg kg^{-1} treatments. The rate constant for ^{14}C-phenanthrene mineralisation in the aqueous-phase (k_{aq}: 0.72–1.92 d^{-1}) was two orders

214 of magnitude higher than in the sorbed phase (k_{ss}: 0.005–0.009 d^{-1}); k_{aq} was comparable
215 in all amended soils but k_{aq} was usually lower in the 10,000 mg kg^{-1} treatment. The
216 predicted t_0 values were comparable to the measured lag phases.
217
218 *3.3. Effect of diesel concentration and soil contact time on ^{14}C-hexadecane*
219 *mineralisation*
220 Mineralisation of ^{14}C-hexadecane in the unamended and diesel-amended soils was very
221 rapid with virtually no lag phases (usually <0.5 d) and rates peaked by the first day of
222 mineralisation (Table 3 and Figure S2). Generally, there were no significant differences
223 ($P > 0.05$) in the lag phases, fastest rates or the T_{max} and the overall extents of
224 mineralisation between the unamended soil and 1–100 mg kg^{-1} treatments at any of the
225 soil–diesel contact times. Higher diesel concentrations (100–10,000 mg kg^{-1}) had
226 significant effects ($P < 0.05$) on ^{14}C-hexadecane mineralisation after 1 and 21 d soil–
227 diesel contact times; the lag phases were longer while fastest rates and extents lower.
228 As soil–diesel contact time increased further, the effects diminished; extents of
229 mineralisation being generally comparable ($P > 0.05$) between the unamended soils
230 and all amended soils.
231 The results of the modelled kinetic parameters for ^{14}C-hexadecane mineralisation are
232 presented in Table 5. A significant portion of the added ^{14}C-hexadecane mineralised to
233 $^{14}CO_2$ was in the aqueous phase (A_0): averaged 69% in the unamended soil and ranged
234 from 71 to 78% in the 1–10,000 mg kg^{-1} treatments. The rate constant for ^{14}C-
235 hexadecane mineralisation in the aqueous-phase (k_{aq}: 0.38–0.44 d^{-1}) was only an order
236 of magnitude higher than in the sorbed phase (k_{ss}: 0.013–0.014 d^{-1}); k_{aq} was comparable
237 in all amended soils but k_{aq} was usually lower in the 10,000 mg kg^{-1} treatment. The
238 predicted t_0 values were comparable to the measured lag phases. The graphical

239 representation of the model fit to the mineralisation data of phenanthrene and
240 hexadecane is available as supplementary material (Figure S1 and S2); the quality of
241 the fit was indicated by the strong $r^2 \geq 0.990$ ($P < 0.001$).
242
243 *3.4. Comparison of the evolution of hydrocarbon catabolic activities*
244 To gain a better understanding of the impact of diesel concentration and soil contact
245 time on the evolution of hydrocarbon catabolic activity, a time-course evolution of ^{14}C-
246 hydrocarbon catabolic activity was determined using the concentration effect (C_{effect})
247 parameter (Figures 1 and 2). The C_{effect} is defined as the ratio of mineralisation rates in
248 a diesel-amended soil to the rates in the unamended soil. An increase (>1) or a decrease
249 (<1) in the C_{effect} indicates enhancing or repressing effect, respectively. In the context
250 of this paper, evolution is used to describe the initiation and expression of catabolic
251 activity within microbial community in response to the presence of a substrate. The
252 C_{effect} profiles presented clearer graphics of the effects on the evolution of catabolic
253 activities as soil–diesel contact time increased. The degree to which phenanthrene
254 catabolic activity was enhanced in the amended soils and the period it remained high
255 above that in the unamended soil are related to the concentration and contact time of
256 diesel in soil (Figure 1). For example, the magnitude of the evolution decreased with
257 increasing diesel concentration from 10 to 1000 mg kg^{-1} at any of the sampling times.
258 In general, the enhancing effect of diesel on the evolution of phenanthrene catabolic
259 activity increased to maxima in all amended soils after 21 d and gradually decline
260 thereafter. Though statistically insignificant ($P > 0.05$), the C_{effect} profiles showed
261 patterns of gradual shifts from repressive on to enhancing effects on the evolution of
262 ^{14}C-hexadecane catabolic activity as contact time increased (Figure 2). For instance,
263 evolution of hexadecane catabolic activity was marginally repressed in the presence of

264 diesel along the concentration-gradient after 1 and 21 d contact times. With further
265 incubation, evolution of hexadecane catabolic activity was slightly enhanced
266 particularly in the 10–1000 mg kg^{-1} treatments.
267
268 **4. Discussion**
269 Similar to this present study, ^{14}C-radiorespirometric assays have been widely employed
270 to assess the development of indigenous catabolic activity towards a target HOC. (e.g.,
271 Bauer and Capone, 1985; Grosser et al., 1995; Carmichael et al., 1997; Reid et al.,
272 2001; Macleod and Semple, 2002; Stroud et al., 2009; Couling et al., 2010). This type
273 of respirometric system allows for the complete distribution of the added contaminants
274 in the soil slurry and ensures optimal microbe–contaminant contact to produce faster
275 mineralisation (Derz et al., 2006). The measurement of indigenous catabolic activity is
276 important in the investigation of contaminant fate, behaviour, ecological risk and
277 bioremediation potential of contaminated soils (Reid et al., 2001). This study was
278 designed to comparatively assess the effect of increasing diesel concentration and soil
279 contact time on the evolution of indigenous catabolic activity towards two model PAHs
280 (phenanthrene and hexadecane). It was also to provide further understanding of the
281 influence of chemical structure–biodegradability relationship on the development of
282 indigenous catabolic activity towards a target hydrocarbon.
283 Consistent with previous work by Stroud et al. (2007a) and Couling et al. (2010), this
284 present study also demonstrated that the potential to degrade and mineralise
285 phenanthrene or hexadecane by the indigenous microbiota was high in the soil
286 investigated. The rapid and extensive mineralisation of ^{14}C-phenanthrene as well as the
287 relatively large number of phenanthrene degraders in the unamended soil can be related
288 to microbial adaptation through prior exposure; phenanthrene contributed ca. 60% to

289 the background ∑PAHs in the soil. In a study of the PAH degradation capacity of 13
290 soils ranging from pristine locations (∑PAHs ~ 0.1 mg kg^{-1}) to heavily polluted
291 industrial sites (∑PAHs ~ 400 mg kg^{-1}), the densities of phenanthrene degraders and
292 their catabolic activity was observed to reflect previous exposure (Johnsen and
293 Karlson, 2005). Adaptation through pre-exposure to a PAH is thought to promote the
294 development catabolic potential within microbial community and the induction of the
295 specialised enzymes in the event of subsequent exposures (Jan Roelof van der, 2006).
296 Hexadecane was not detected at background level in this soil; however, there were
297 larger numbers of indigenous bacteria able to mineralise ^{14}C-hexadecane and its
298 mineralisation proceeded with apparently no lag phase and at much faster rate and to a
299 greater extent compared to ^{14}C-phenanthrene. This is remarkable in that hexadecane is
300 more hydrophobic with a lower aqueous solubility (0.0009 *vs.* 1.1 mg l^{-1}) and more
301 lipophilic with a higher partitioning coefficient (log K_{ow} 9.1 *vs.* 4.16) than
302 phenanthrene (Stroud et al., 2007b). The almost immediate and initially faster rate of
303 mineralisation of hexadecane can be attributed to the constitutive nature of the
304 enzymatic pathways often used for hexadecane biodegradation in contrast to the
305 inducible enzymatic system that is required for the biodegradation of phenanthrene
306 (Stroud et al., 2007b). In addition, the results from the model fitted to the
307 mineralisation data indicated that substantial mineralisation of the ^{14}C-hydrocarbons
308 occurred also in the soil inter-phase surfaces; this being greater for ^{14}C-hexadecane
309 (22–29%) than ^{14}C-phenanthrene (12–22%). This may explain the higher extent of ^{14}C-
310 hexadecane mineralisation recorded in this study. Although there is a large collection
311 of studies in the literature in support of microbial uptake of HOCs occurring mainly in
312 the aqueous phase, there is an increasing body of evidence pointing also to the direct
313 uptake from inter-phase surfaces (Baboshin and Golovleva, 2012).

314 Studies have evidenced that microorganisms can adapt via certain specialised
315 mechanisms to enhance the passive uptake of poorly-soluble HOCs like hexadecane;
316 these include the high hydrophobicity of microbial cell walls to facilitate direct cell
317 contact with the HOCs and/or the production of biosurfactants to promote higher
318 solubilisation of the HOCs (Bouchez-Naïtali et al., 2001; Stroud et al., 2007b;
319 Bouchez-Naitali and Vandecasteele, 2008). Rapid and extensive mineralisation of
320 hexadecane within hours was linked to the direct interfacial uptake by strains of
321 *Rhodococcus* (Bouchez-Naïtali et al., 2001), and to biosurfactant production by
322 *Pseudomonas* strains (Bouchez-Naitali and Vandecasteele, 2008). The potentially
323 biodegradable fractions of HOCs in soil which can be quantified chemically in the
324 laboratory (i.e. the bioaccessible fraction) consist of the fraction that may readily
325 desorb from soil to and/or is present in the aqueous phase (Semple et al., 2007). In the
326 case of straight-chain *n*-alkanes, studies to predict bioaccessibility and therefore the
327 extent of mineralisation have showed that the desorbed fraction is always less than that
328 of the microbially degraded fraction, indicating that the direct uptake of this class of
329 hydrocarbons from the soil inter-phase surfaces without prior desorption to the aqueous
330 phase is an important contributor to their biodegradation (Huesemann et al., 2003,
331 2004).
332 The rate of microbial degradation of HOCs in soils is thought to be a function of three
333 variables: (i) the availability of the chemicals to the competent microorganisms, (ii) the
334 population density of these microorganisms and (iii) the catabolic activity level of these
335 organisms (Sepic et al., 1995). Results of the C_{effect} profiles suggested that the
336 limitation to a significantly greater extent of mineralisation in the 10–1000 mg kg^{-1}
337 treatments, as compared to the unamended soil, was due to the decreased ^{14}C-
338 hydrocarbon bioaccessibility (in the aqueous phase) rather than the inability of diesel to

339 support higher proliferation of phenanthrene-degrading bacteria and/or enhance their
340 catabolic activity. It was clearly demonstrated through a series of experiments in a soil
341 with high catabolic ability for phenanthrene that the termination of mineralisation was
342 due to the removal of the bioaccessible fraction of the chemical rather than decreasing
343 cellular activity or cell death (Semple et al., 2006). Consistent with the findings of
344 previous studies, the results of this present study also showed that the development of
345 indigenous catabolic activity toward PAHs can be completely suppressed or
346 progressively repressed in soils contaminated with high concentrations of diesel.
347 Swindell and Reid (2007) showed that phenanthrene catabolism was retarded in two
348 dissimilar soils amended to 20,000 mg kg^{-1} and in a recent unpublished research from
349 the authors' laboratory, it was highlighted that naphthalene catabolic activity was
350 progressively repressed in a 10,000 mg kg^{-1} diesel-amended soil.
351 In comparison, the C_{effect} profiles indicated that diesel enhanced the initiation and
352 expression of microbial catabolic activity towards phenanthrene but not hexadecane.
353 This is not surprising since a constitutive rather than an inducible enzymatic system is
354 often required for hexadecane biodegradation (Bardi et al., 2000; Stroud et al., 2007b).
355 Lastly, it is suggested that analysing mineralisation data using the C_{effect} parameter can
356 provide additional practical insights to the spatial and/or temporal localisation of
357 indigenous catabolic activity at sites with varying contaminant concentrations.
358
359 *Relevance of finding*
360 In summary, this study has demonstrated that, over a range of concentrations (0–10,000
361 mg kg^{-1}) diesel did not affect the evolution of hexadecane catabolic activity, whereas it
362 either enhanced or repressed phenanthrene catabolic activity depending on the initial
363 pre-exposure concentration and soil contact time. Either occurring naturally (natural

attenuation) or enhanced (bioremediation), microbial degradation is a key process for the decontamination of polluted sites. Hence, the potential variations in microbial catabolic activity over a relatively small area as a result of the high heterogeneity (sometimes ranging over several orders of magnitude) in petroleum hydrocarbon concentrations at polluted sites may make the assessment and/or design of bespoke bioremediation programme more challenging.

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505 **Tables**

506 Table 1: Colony forming units (CFUs) of hydrocarbon-degrading bacteria in
507 unamended soil and soils amended with increasing diesel concentration after different
508 soil–diesel contact times. Values are the means (n = 3) ± standard error of the mean
509 (SEM).

Time (d)	Concentration (mg kg^{-1})	Hydrocarbon degrader numbers (CFUs g^{-1})	
		Phenanthrene	Hexadecane
1	0	1.62E+06 ± 4.37E+04	1.91E+07 ± 1.51E+04
	1	1.66E+06 ± 1.91E+04	1.78E+07 ± 3.16E+05
	10	4.27E+06 ± 1.32E+04	3.80E+07 ± 2.34E+05
	100	3.24E+06 ± 2.19E+03	3.47E+07 ± 3.72E+05
	1000	8.32E+06 ± 1.55E+04	1.38E+07 ± 2.75E+05
	10,000	1.78E+06 ± 1.95E+04	1.41E+07 ± 1.74E+06
21	0	2.45E+06 ± 5.89E+04	2.88E+07 ± 1.41E+05
	1	2.04E+06 ± 3.72E+04	2.34E+07 ± 2.69E+05
	10	3.63E+06 ± 6.92E+05	3.89E+07 ± 7.41E+04
	100	3.89E+06 ± 8.32E+04	4.68E+07 ± 2.88E+05
	1000	4.47E+07 ± 2.69E+04	4.90E+07 ± 4.57E+05
	10,000	3.63E+07 ± 7.41E+03	2.45E+07 ± 2.95E+05
42	0	2.88E+06 ± 5.62E+03	1.91E+07 ± 1.45E+05
	1	1.82E+06 ± 1. 20E+04	2.19E+07 ± 2.14E+05
	10	3.02E+06 ± 1.23E+04	3.55E+07 ± 2.57E+05
	100	6.46E+06 ± 1.07E+03	3.55E+07 ± 1.29E+05
	1000	6.17E+07 ± 1.20E+04	2.82E+07 ± 1.95E+05
	10,000	3.24E+07 ± 3.02E+05	2.57E+07± 4.27E+06
63	0	9.12E+05 ± 1.51E+03	7.41E+06 ± 2.63E+05
	1	1.70E+06 ± 2.69E+04	5.89E+07 ± 4.68E+05
	10	3.89E+06 ± 2.04E+04	4.17E+07 ± 5.50E+04
	100	6.46E+06 ± 7.24E+03	7.24E+07 ± 9.55E+05
	1000	6.61E+07 ± 2.51E+04	5.75E+07 ± 3.55E+05
	10,000	3.98E+07 ± 7.08E+05	5.37E+07 ± 2.88E+05

510

511 Table 2: Effect of increasing diesel concentration and soil contact time on indigenous

512 microbial mineralisation of ^{14}C-phenanthrene

Time (d)	Concentration (mg kg^{-1})	Lag time (d)	Fastest rate (% d^{-1}); T_{max} (d)	Overall extent (%)
1	0	3.39 ± 0.04 ^{a§A‡}	16.13 ± 0.59 ^{aA} (5)†	52.15 ± 0.44 ^{aA}
	1	3.33 ± 0.02 ^{aA}	17.47 ± 1.49 ^{aA} (5)	55.14 ± 1.05 ^{aA}
	10	3.14 ± 0.01 ^{aA}	29.13 ± 1.51 ^{bA} (4–5)	56.56 ± 2.26 ^{abA}
	100	3.85 ± 0.23 ^{aA}	30.58 ± 2.73 ^{bA} (4–5)	59.27 ± 2.21 ^{abA}
	1000	5.31 ± 0.21 ^{bA}	23. 35 ± 3.81 ^{cA} (5–9)	64.10 ± 0.71 ^{bA}
	10,000	>16.95$^{\#}$ ^{cA}	1.83 ± 1.50 ^{dA} (>18)	5.88 ± 2.89 ^{cA}
21	0	5.26 ± 0.16 ^{aA}	19.55 ± 3.01 ^{aA} (6–7)	54.90 ± 0.86 ^{aA}
	1	4.13 ± 0.56 ^{bA}	17.31 ± 0.12 ^{aA} (5–6)	51.53 ± 0.40 ^{aA}
	10	4.21 ± 0.73 ^{bA}	29.98 ± 3.25 ^{bA} (5–6)	62.77 ± 0.33 ^{bA}
	100	4.06 ± 0.04 ^{bA}	26.00 ± 0.92 ^{bA} (5–6)	55.24 ± 3.42 ^{abA}
	1000	4.19 ± 0.24 ^{bA}	21.74 ± 4.68 ^{abA} (5–6)	50.29 ± 1.67 ^{aB}
	10,000	7.11 ± 0.10 ^{cB}	10.71 ± 0.29 ^{cA} (9–10)	43.10 ± 0.36 ^{cB}
42	0	5.39 ± 0.01 ^{aA}	20.42 ± 0.55 ^{aA} (5–6)	54.00 ± 0.90 ^{aA}
	1	3.81 ± 0.24 ^{bA}	18.41 ± 4.22 ^{aA} (5–6)	53.34 ± 2.76 ^{aA}
	10	4.13 ± 0.10 ^{bA}	20.81 ± 2.63 ^{aB} (6–7)	52.91 ± 0.06 ^{aAB}
	100	3.80 ± 0.17 ^{bA}	21.82 ± 2.72 ^{aB} (5–6)	49.04 ± 2.12 ^{aB}
	1000	4.38 ± 0.25 ^{bA}	23.06 ± 5.82 ^{aA} (5–7)	53.25 ± 0.47 ^{aB}
	10,000	7.54 ± 0.06 ^{cB}	13.82 ± 1.29 ^{bB} (9–10)	42.07 ± 1.35 ^{bB}
63	0	3.72 ± 0.24 ^{aA}	15.96 ± 2.25 ^{aA} (5)	51.60 ± 1.01 ^{aA}
	1	3.24 ± 0.24 ^{aA}	17.95 ± 2.06 ^{aA} (5–6)	50.16 ± 0.82 ^{aA}
	10	3.01 ± 0.34 ^{aA}	18.02 ± 0.48 ^{aB} (5–6)	49.02 ± 1.19 ^{aB}
	100	2.93 ± 0.17 ^{aB}	19.08 ± 2.75 ^{aB} (4–5)	49.77 ± 1.30 ^{aB}
	1000	8.45 ± 0.28 ^{bB}	15.95 ± 1.52 ^{aB} (10)	46.82 ± 2.01 ^{aC}
	10,000	7.13 ± 1.53 ^{bB}	10.82 ± 1.80 ^{bB} (8–11)	31.87 ± 0.19 ^{bC}

513 § Different lower-case letters down the column within each sub-group of "Time" indicate mean values of triplicate
514 samples that are significantly different ($P < 0.05$).
515 ‡ Different upper-case letters down the column for corresponding "Concentration" across groups of "Time" indicate
516 mean values of triplicate samples that are significantly different ($P < 0.05$).
517 † Values in parentheses are time taken for mineralisation to reach fastest rates in days ($n = 3$). Where a single value
518 is shown, all triplicate samples reached fastest rates by the date shown; where a range is shown, data reflect the
519 variability in the triplicate measurements.
520 # Only one of the triplicate samples reached >5% mineralisation before the end of the experiment
521
522

523 Table 3: Effect of increasing diesel concentration and soil contact time on indigenous

524 microbial mineralisation of ^{14}C-*n*-hexadecane

Time (d)	Concentration (mg kg^{-1})	Lag time (d)	Fastest rate (% d^{-1}); T_{max} (d)	Overall extent (%)
1	0	0.15 ± 0.01 ^{a§A‡}	34.10 ± 1.66 ^{aAB} (1)†	68.43 ± 2.14 ^{aA}
	1	0.16 ± 0.00 ^{aA}	32.09 ± 0.91 ^{aA} (1)	66.79 ± 0.55 ^{aA}
	10	0.16 ± 0.00 ^{aA}	31.60 ± 0.80 ^{aA} (1)	62.14 ± 1.08 ^{aA}
	100	0.18 ± 0.03 ^{aA}	28.93 ± 4.09 ^{aA} (1)	59.99 ± 3.05 ^{abA}
	1000	0.19 ± 0.00 ^{aA}	25.66 ± 0.57 ^{aA} (1)	56.02 ± 4.56 ^{bA}
	10,000	0.45 ± 0.00 ^{bA}	15.13 ± 0.00 ^{bA} (3)	52.72 ± 0.13 ^{bA}
21	0	0.13 ± 0.01 ^{aA}	40.68 ± 3.91 ^{aB} (1)	70.49 ± 6.13 ^{aA}
	1	0.15 ± 0.03 ^{aA}	36.13 ± 6.09 ^{aA} (1)	71.55 ± 3.42 ^{aA}
	10	0.12 ± 0.01 ^{aA}	44.27 ± 4.55 ^{abB} (1)	60.22 ± 5.21 ^{abA}
	100	0.11 ± 0.00 ^{aA}	47.05 ± 1.54 ^{bC} (1)	60.12 ± 2.61 ^{abA}
	1000	0.18 ± 0.03 ^{abA}	28.89 ± 3.88 ^{cA} (1)	51.20 ± 6.00 ^{bA}
	10,000	0.24 ± 0.06 ^{bB}	22.76 ± 4.30 ^{cB} (1)	53.38 ± 1.99 ^{bA}
42	0	0.18 ± 0.02 ^{aA}	26.02 ± 2.96 ^{aA} (1)	57.65 ± 0.94 ^{aB}
	1	0.17 ± 0.02 ^{aA}	30.40 ± 4.51 ^{aA} (1)	59.33 ± 4.11 ^{aB}
	10	0.18 ± 0.02 ^{aA}	28.07 ± 2.17 ^{aA} (1)	58.59 ± 0.80 ^{aA}
	100	0.13 ± 0.01 ^{bA}	40.11 ± 2.03 ^{bB} (1)	71.28 ± 0.49 ^{bB}
	1000	0.14 ± 0.01 ^{bB}	37.02 ± 1.44 ^{bB} (1)	64.50 ± 1.60 ^{abB}
	10,000	0.18 ± 0.02 ^{aB}	28.53 ± 2.25 ^{aB} (1)	59.50 ± 1.39 ^{aB}
63	0	0.12 ± 0.01 ^{aA}	41.44 ± 2.35 ^{aB} (1)	76.49 ± 1.98 ^{aC}
	1	0.11 ± 0.01 ^{aA}	46.93 ± 2.38 ^{abA} (1)	73.52 ± 4.50 ^{aA}
	10	0.11 ± 0.01 ^{aA}	47.35 ± 2.40 ^{abB} (1)	71.74 ± 3.65 ^{aB}
	100	0.10 ± 0.00 ^{aA}	51.74 ± 2.27 ^{abC} (1)	78.28 ± 3.92 ^{aC}
	1000	0.09 ± 0.00 ^{aC}	55.71 ± 2.73 ^{bC} (1)	81.80 ± 2.10 ^{aC}
	10,000	0.24 ± 0.03 ^{bB}	21.69 ± 3.15 ^{cB} (1)	77.40 ± 4.23 ^{aC}

525 § Different lower-case letters down the column within each sub-group of "Time" indicate mean values of triplicate
526 samples that are significantly different ($P < 0.05$).
527 ‡ Different upper-case letters down the column for corresponding "Concentration" across groups of "Time" indicate
528 mean values of triplicate samples that are significantly different ($P < 0.05$).
529 † Values in parentheses are time taken for mineralisation to reach fastest rates in days ($n = 3$). Where a single value
530 is shown, all triplicate samples reached fastest rates by the date shown; where a range is shown, data reflect the
531 variability in the triplicate measurements.

532

533 Table 4: Modelled kinetic parameters for mineralisation of ^{14}C-phenanthrene‡

Time (d)	Concentration (mg kg^{-1})	A (%)	k_{aq} (d^{-1})	t_0 (d)	k_{ss} (10^{-2} d^{-1})	r^2
1	0	37.20 (1.25)	0.91 (0.08)	4.35 (0.05)	0.86 (0.09)	0.999
	1	43.28 (0.82)	0.86 (0.05)	4.28 (0.03)	0.68 (0.06)	0.999
	10	45.82 (0.58)	0.42 (0.05)	3.70 (0. 04)	0.63 (0.05)	0.999
	100	41.07 (1.29)	0.41 (0.23)	4.39 (0.35)	1.08 (0.10)	0.997
	1000	55.97 (1.44)	1.16 (0.08)	7.08 (0.06)	0.46 (0.09)	0.998
	10,000#					
21	0	45.97 (2.86)	1.92 (0.15)	5.86 (0.06)	0.51 (0.17)	0.999
	1	39.86 (4.07)	1.06 (0.24)	6.35 (0.09)	0.62 (0.24)	0.997
	10	44.74 (3.46)	1.07 (0.19)	5.05 (0.09)	1.01 (0.23)	0.993
	100	38.38 (0.92)	0.63 (0.06)	4.97 (0.03)	0.98 (0.07)	0.999
	1000	36.86 (1.48)	1.15 (0.09)	5.24 (0.05)	0.77 (0.10)	0.999
	10,000	36.64 (1.35)	1.37 (0.09)	8.20 (0.06)	0.31 (0.08)	0.999
42	0	44.17 (1.63)	0.72 (0.08)	6.78 (0.05)	0.58 (0.11)	0.998
	1	40.83 (1.78)	1.19 (0.10)	5.17 (0.06)	0.71 (0.12)	0.998
	10	41.99 (1.33)	1.16 (0.07)	5.53 (0.04)	0.62 (0.09)	0.999
	100	37.67 (0.74)	0.92 (0.05)	4.59 (0.03)	0.67 (0.05)	0.999
	1000	36.80 (1.91)	0.89 (0.12)	5.18 (0.07)	0.90 (0.14)	0.997
	10,000	37.08 (0.93)	1.00 (0.06)	8.75 (0.04)	0.26 (0.06)	0.999
63	0	40.53 (1.17)	1.27 (0.08)	4.15 (0.04)	0.65 (0.08)	0.999
	1	39.32 (0.82)	1.17 (0.05)	4.46 (0.03)	0.62 (0.06)	0.999
	10	36.72 (0.64)	1.10 (0.05)	4.22 (0.03)	0.86 (0.05)	0.999
	100	33.76 (0.67)	0.93 (0.04)	3.88 (0.03)	0.77 (0.05)	0.999
	1000	42.76 (1.01)	1.13 (0.06)	9.34 (0.04)	0.17 (0.06)	0.999
	10,000#					

534 ‡ Values represent mean (SEM) of triplicate readings. A_0 is the asymptotic cumulative $^{14}CO_2$ evolved
535 (%); k_{aq} is the rate constant in aqueous phase (d^{-1}); t_0 is the time in days at the inflection point of the
536 modified Gompertz model curve where mineralisation transit from linear to exponential rate; k_{ss} is rate
537 constant in the sorbed phase (d^{-1}).
538 # Values not provided; model did not fit to mineralisation data
539

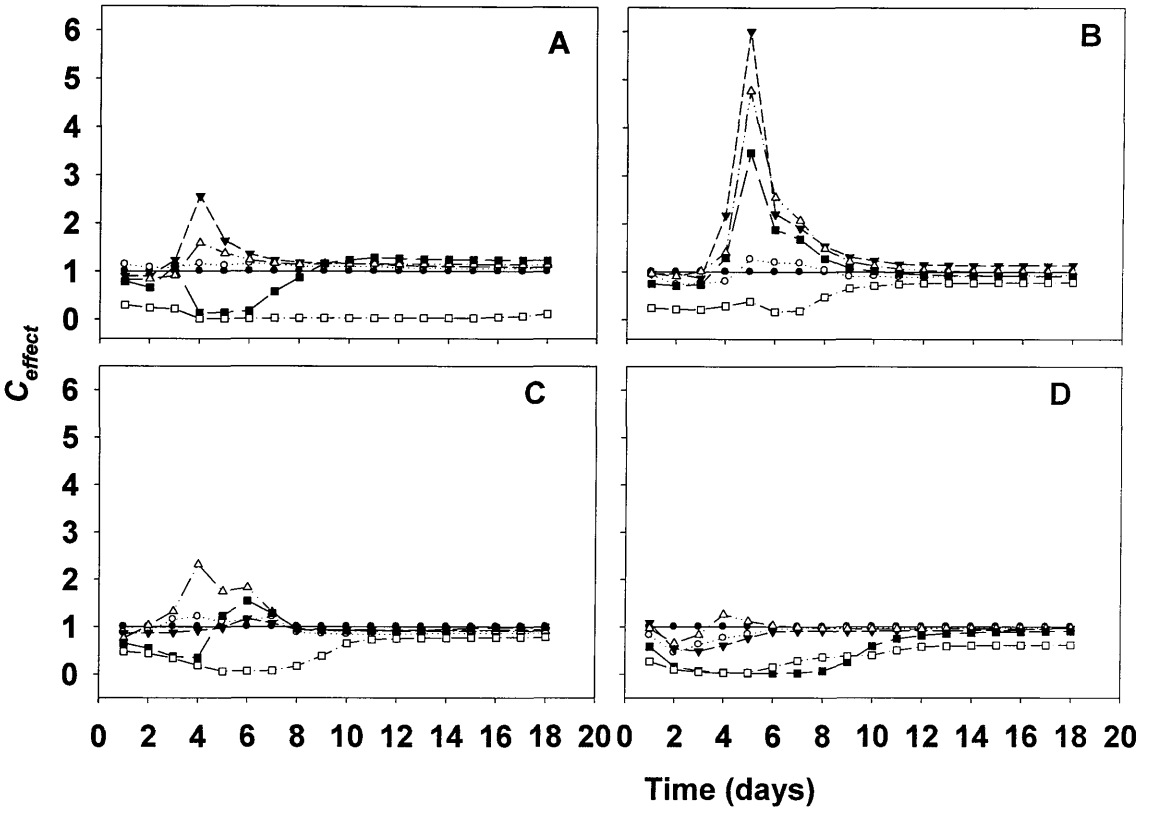
Table 5: Modelled kinetic parameters for mineralisation of ^{14}C-*n*-hexadecane‡

Time (d)	Concentration (mg kg^{-1})	A (%)	k_{aq} (d^{-1})	t_0 (d)	k_{ss} (10^{-2} d^{-1})	r^2
1	0	46.07 (1.22)	0.44 (0.08)	0.56 (0.09)	1.40 (0.12)	0.992
	1	44.12 (1.26)	0.46 (0.09)	0.57 (0.09)	1.40 (0.13)	0.991
	10	43.31 (1.08)	0.44 (0.08)	0.57 (0.08)	1.18 (0.11)	0.992
	100	40.15 (1.16)	0.47 (0.09)	0.58 (0.09)	1.24 (0.12)	0.991
	1000	37.21 (1.06)	0.51 (0.09)	0.61 (0.08)	1.14 (0.11)	0.992
	10,000	42.22 (1.76)	1.22 (0.13)	1.58 (0.09)	0.62 (0.15)	0.994
21	0	51.27 (1.56)	0.38 (0.09)	0.52 (0.12)	1.22 (0.16)	0.990
	1	51.08 (1.65)	0.35 (0.10)	0.49 (0.14)	1.29 (0.17)	0.988
	10	34.01 (1.69)	0.62 (0.15)	0.64 (0.12)	1.19 (0.17)	0.985
	100	36.06 (1.10)	0.37 (0.09)	0.51 (0.12)	0.94 (0.11)	0.990
	1000	50.54 (0.33)	0.23 (0.05)	0.42 (0.11)	0.56 (0.03)	0.999
	10,000	51.66 (0.67)	0.30 (0.05)	0.47 (0.09)	0.52 (0.07)	0.997
42	0	36.56 (1.46)	0.51 (0.11)	0.59 (0.11)	1.29 (0.14)	0.989
	1	40.46 (1.18)	0.42 (0.09)	0.55 (0.10)	1.15 (0.12)	0.993
	10	38.83 (1.04)	0.46 (0.08)	0.58 (0.08)	1.18 (0.10)	0.994
	100	50.74 (1.29)	0.38 (0.08)	0.52 (0.10)	1.25 (0.13)	0.993
	1000	46.18 (1.12)	0.36 (0.08)	0.52 (0.11)	1.11 (0.11)	0.994
	10,000	45.06 (1.72)	0.61 (0.11)	0.66 (0.09)	0.91 (0.16)	0.989
63	0	55.01 (1.19)	0.41 (0.07)	0.55 (0.08)	1.33 (0.13)	0.994
	1	56.76 (0.89)	0.33 (0.06)	0.50 (0.09)	1.02 (0.10)	0.996
	10	55.83 (0.72)	0.31 (0.05)	0.49 (0.09)	0.95 (0.08)	0.997
	100	61.58 (0.98)	0.32 (0.06)	0.49 (0.10)	1.03 (0.11)	0.996
	1000	64.72 (0.86)	0.30 (0.06)	0.48 (0.10)	1.04 (0.09)	0.997
	10,000	55.35 (4.47)	1.47 (0.28)	1.23 (0.19)	1.36 (0.36)	0.986

‡ Values represent mean (SEM) of triplicate readings. A_0 is the asymptotic cumulative $^{14}CO_2$ evolved (%); k_{aq} is the rate constant in aqueous phase (d^{-1}); t_0 is the time in days at the inflection point of the modified Gompertz model curve where mineralisation transit from linear to exponential rate; k_{ss} is rate constant in the sorbed phase (d^{-1}).

545 **Figures**

546



547
548

549 Figure 1: Evolution of phenanthrene catabolic activity in soil amended with diesel to 0

550 (●), 1 (○), 10 (▼), 100 (Δ), 1000 (■) and 10,000 mg kg^{-1} (□) after (A) 1, (B) 21, (C) 42

551 and (D) 63 d of contact.

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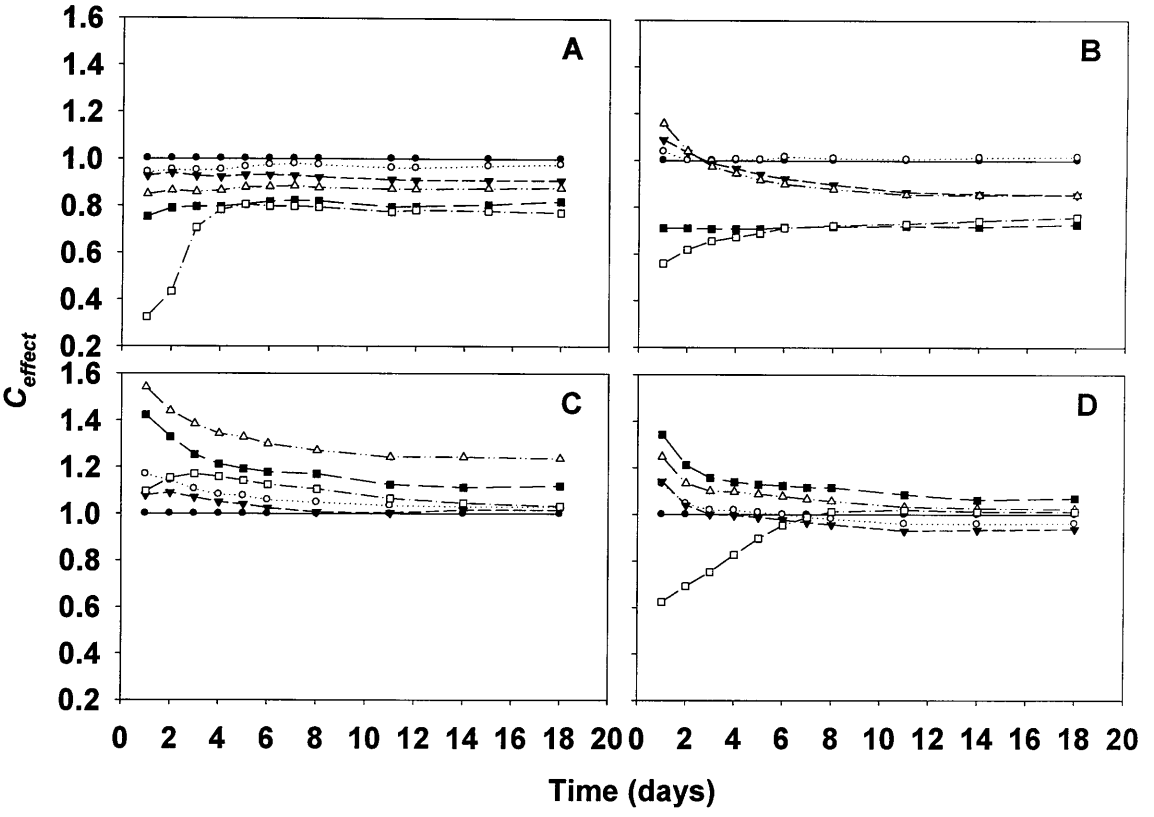

555
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557

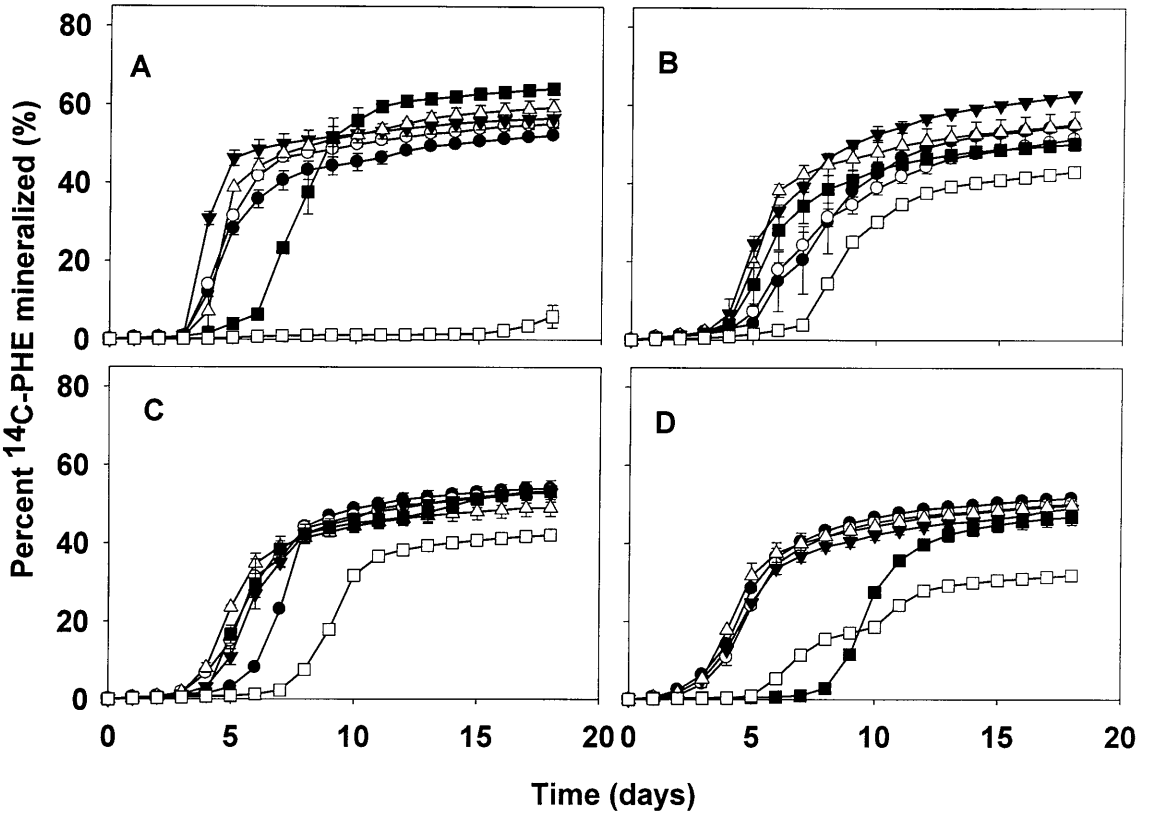
558 Figure 2: Evolution of *n*-hexadecane catabolic activity in soil amended with diesel to 0

559 (●), 1 (○), 10 (▼), 100 (▽), 1000 (■) and 10,000 mg kg^{-1} (□) after (A) 1, (B) 21, (C)

560 42, and (D) 63 d of contact.

561 **Supplementary data**

562



563
564

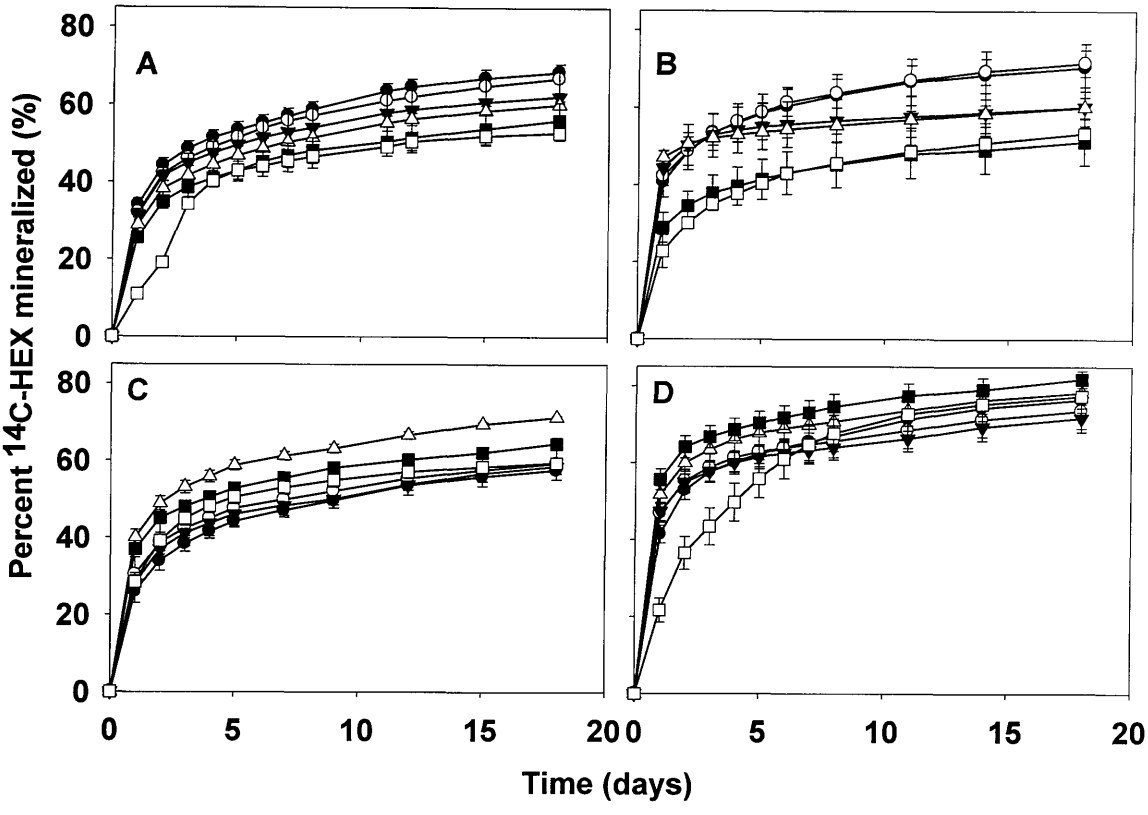
565 Figure S1: Mineralisation of ^{14}C-phenanthrene in soil amended with diesel to 0 (●), 1

566 (○), 10 (▼), 100 (Δ), 1000 (■) and 10,000 mg kg^{-1} (□) after (A) 1 (B) 21 (C) 42 and

567 (D) 63 d of contact.

568

569



570
571

572 Figure S2: Mineralisation of ^{14}C-*n*-hexadecane in soil amended with diesel to 0 (●), 1

573 (○), 10 (▼), 100 (Δ), 1000 (■) and 10,000 mg kg^{-1} (□) after (A) 1, (B) 21, (C) 42 and

574 (D) 63 d of contact.

Paper IV

Effect of diesel oil concentration on microbial utilisation of ^{14}C-glucose in soil

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Abstract

Radiorespirometry was used to investigate the effect of diesel, along a concentration-gradient (applied at log rates between 1 and 10,000 mg kg^{-1}), on the metabolic response of the extant soil microflora to fresh input of labile carbon substrate. After 1, 21, 42 and 63 d soil–diesel contact times, microbial utilisation of ^{14}C-glucose was quantified as $^{14}CO_2$ and ^{14}C-biomass during 120-h incubation. Results indicated that low diesel concentrations (1–100 mg kg^{-1}) had negligible effects, whereas higher concentrations (1000–10,000 mg kg^{-1}) shifted the pattern of ^{14}C-glucose utilisation with greater allocation to ^{14}C-biomass as soil–diesel contact time increased. This was complemented by the relatively greater increases in the abundance of phenanthrene- and octacosane-degrading bacteria after ^{14}C-glucose mineralisation in the 1000–10,000 mg kg^{-1} soils. It is suggested that the actively growing hydrocarbon-degrading microorganisms in the highly-polluted soils are more likely to preferentially metabolise fresh input of easier-to-degrade carbon substrate for biosynthesis rather than for respiratory purposes.

Capsule:

Diesel concentration affects the metabolic response of extant soil microflora to fresh input of labile carbon substrate.

Keywords: *Biophysical quotient; Concentration effect; Diesel oil; Microbial respiratory activity; Microbial biomass; Mineralisation; Substrate induced respiration*

1. Introduction

Pollution of soil by spillage of crude or refined petroleum products, such as diesel oil, and by disposal of industrial wastes often produce changes in the soil microbial communities, including acute and, sometimes chronic shifts in their abundance, diversity and metabolic processes, as well as genetic characteristics (Phillips, *et al.*, 2000, Yrjälä, *et al.*, 2010, Perez-Leblic, *et al.*, 2012). These pollutants often consist of hydrophobic organic chemicals, including aliphatic and polycyclic aromatic hydrocarbons, which have low aqueous solubility, low volatility, and high affinity for particulate matter – properties that tend to make them accumulate and persist in soil (Bamforth & Singleton, 2005). Because of the toxic, recalcitrant, mutagenic and carcinogenic nature of some of these petroleum hydrocarbons, several studies have been carried out in order to effectively decontaminate and/or detoxify polluted sites (Xu & Lu, 2012, Lladó, *et al.*, 2013, Pelaez, *et al.*, 2013).

Microorganisms are the principal agents for decontamination of petroleum hydrocarbon-polluted soils but they are also sensitive to and may be negatively affected by the pollutants (Killham, 1985, Boucard, *et al.*, 2008). In extreme situations, prolonged modifications to microbial diversity and activity, such as nutrient availability and cycling, may cause deterioration or irreversible distortion to ecosystem functioning (Nannipieri, *et al.*, 2003). In term of the overall functioning of the soil ecosystem, microorganisms play a vital role in that most of the energy flow passes through heterotrophic soil microflora (Nannipieri, *et al.*, 2003). Hence, alterations to microbial properties (e.g., abundance and diversity, metabolic activity and biomass size), or processes (e.g., nutrient translocation and cycling, regulation of active nutrient pools), have been widely used as indices of the severity of a perturbation on soil health and quality, and of the restoration afterwards (Griffiths, *et al.*, 2001). In particular,

63 because of their relatively high sensitivity and rapid responses to physical or chemical
64 stress, microbial biomass and/or its activity (e.g. basal or substrate-induced respiration)
65 have been used to reflect the effect of perturbations (Killham, 1985, Reid, *et al.*, 2000,
66 Mikkonen, *et al.*, 2012).
67 Further, the use of parameters that link microbial biomass to its activity, such microbial
68 metabolic quotient (qCO_2), biophysical quotient (BQ) or microbial yield coefficient
69 (*Yc*) is thought to provide additional valuable information compared to the respiration
70 rate or the amount of biomass alone (Anderson & Domsch, 1985, van Beelen &
71 Doelman, 1997). These parameters have been used in various studies ranging from
72 investigation of the effect of environmental variables and land management to impact
73 of pollutant on microbial activity, and have been explored as indices of ecosystem
74 disturbance and development or as measures of metabolic efficiency (Wardle & Ghani,
75 1995). ^{14}C-Radiorespirometry is a robust technique and has been used by several
76 investigators to the measure the efficiency of utilising a labelled substrate by
77 indigenous microorganisms under various pollution conditions in soils (Killham, 1985,
78 Phillips, *et al.*, 2000, Reid, *et al.*, 2000, Boucard, *et al.*, 2008, Towell, *et al.*, 2011). In
79 polluted soil environments where there are various carbon substrates, this technique is
80 apt to trace the fate of a target carbon substrate; for instance, Boucard, *et al.* (2008)
81 used ^{14}C-glucose to evaluate the impact of sheep dip formulations on microbial
82 biomass and its activity in soil.
83 In this paper, the effect of diesel, applied to soil at log loading rates from 1 to 10,000
84 mg kg^{-1}, on the metabolic response of the extant soil microflora to fresh input of labile
85 carbon substrate was investigated by ^{14}C-radiorespirometry.
86

2. Materials and methods

2.1. Soil properties and soil amendment with diesel oil

The Dystric Cambisol was collected (A horizon; 5–20 cm) from a pasture field at Myerscough Agricultural College (grid reference SD496402), Lancashire, UK. The soil is a sandy loam (56.63% sand, 24.96% silt, 19.41% clay), with 17 ± 0.09 g kg^{-1} total organic carbon, 1.4 ± 0.1 g kg^{-1} nitrogen, and a pH (in dH_2O) of 6.53 (Couling, *et al.*, 2010). The soil has no known history of exposure to anthropogenic petroleum hydrocarbons (30 μg ∑16 USEPA PAH kg^{-1}). The soil was air-dried at 21 ± 1 °C for ~48 h, sieved (≤2-mm) and stored at 4 °C until needed. A week before the start of experiments, soil samples were conditioned at 21 °C in the dark to reduce the priming effect and ensure the endogenous microbial respiration is stabilised. Amendment followed the method described by Doick, *et al.* (2003); briefly, different amounts of diesel oil (specific gravity 0.85, C-content 87%; from a local BP fuel station in Lancaster, UK) were applied to soil at concentrations of 0, 1, 10, 100, 1000 and 10,000 mg kg^{-1}; sufficient sterilised Milli-Q water was added to bring soil moisture content to 60% of water holding capacity (WHC). The amended soils were placed in pre-cleaned amber glass jars, loosely sealed and stored in the dark at 21 ± 1 °C to initiate the ageing process and at defined intervals (1, 21, 42 and 63 d) sampled and analysed as described in the following sections.

2.2. Microbial utilisation of ^{14}C-glucose in amended soils

At each sampling time, soil microbial biomass and activity was determined as the metabolic potential of indigenous soil microorganisms to utilise [1-^{14}C]glucose (55.7 mCi $mmol^{-1}$, >99.6%; Sigma–Aldrich, UK) as a carbon source for respiration (evolved as $^{14}CO_2$) and/or for growth (assimilated as ^{14}C-biomass). The substrate-induced

112 respiration (SIR) coupled to chloroform fumigation-extraction (CFE) technique was
113 used to quantify the mineralisation of ^{14}C-glucose and microbial ^{14}C-biomass
114 incorporation (Vance, *et al.*, 1987, Sparling & West, 1988). A preliminary experiment
115 was carried out to define the glucose concentration that will produce an optimal
116 respiratory response (V_{max}) in the soil (data not shown).
117 The radiorespirometric assays consisted of soil (20 ± 0.2 g) and 10 ml of 3 mM glucose
118 solution (1080 μg g^{-1}) with an associated ^{14}C-glucose activity of *ca.* 40 Bq g^{-1} (Reid, *et*
119 *al.*, 2001). A substrate concentration in excess of what can trigger a maximum initial
120 respiratory response is recommended for respirometric assays (Aira & Domínguez,
121 2010). The glucose was initially prepared in a minimal basal salt (MBS) solution
122 composed to adequately provide the essential nutrients and trace elements that can limit
123 ^{14}C-glucose mineralisation (Reid, *et al.*, 2001). A soil:liquid ratio of 2:1 with
124 continuous aeration was adopted to promote rapid and maximum ^{14}C-glucose uptake
125 (Boucard, *et al.*, 2008). Sampling was carried out at 2, 4, 6, 9, 12, 24, 30, 48, 72, 96
126 and 120 h after incubation. The $^{14}CO_2$ trapped was mixed with 5 ml Goldstar liquid
127 scintillation cocktails and quantified by liquid scintillation counting (LSC) (Tri-Carb
128 2300TR LSC; Canberra Packard, UK). The lag phase (time elapsed in hours before
129 mineralisation reached 5%), initial fastest rate, and overall extent of ^{14}C-glucose
130 mineralisation were calculated from the mineralisation data.
131 After the radiorespirometric assays were terminated at 120 h, soil from each
132 respirometer was divided into three portions. The first portion (*ca.* 1 g; oven dried at 30
133 °C, n = 3) was combusted (Packard 307 sample oxidiser, Canberra Packard) to
134 determine the level ^{14}C-activity remaining (residual ^{14}C-activity). The other two
135 portions were processed to estimate the amounts of ^{14}C-glucose incorporated into the
136 microbial biomass (^{14}C-biomass) or loosely-bound to soil matrices by the CFE

137 technique (Vance, *et al.*, 1987). Briefly, the second portion (6 g) was immediately
138 extracted with 0.5 M K_2SO_4 (30 ml) by shaking in an orbital shaker (30 min at 100
139 rpm). The soil solution was centrifuged (3000 x *g*, 30 min), and the supernatant and
140 analysed by LSC, as described previously. The third portion was fumigated with
141 ethanol-free chloroform in a desiccator for 24-h, extracted with 0.5 M K_2SO_4,
142 centrifuged and analysed by LSC. The spent pellets (1 g soil, $n = 3$) from unfumigated
143 and fumigated samples were combusted to determine the amount of ^{14}C-activity left.
144 Marstorp and Witter (1999) has demonstrated that newly synthesised microbial
145 biomass in spiked soils could be estimated by the fumigation-extraction method after 5
146 d of substrate addition. Because of the probable interference of high diesel
147 concentration on the K_2SO_4 extraction efficiency a fixed k_{EC} value as recommended by
148 Vance, *et al.* (1987) was not used; instead, alternative k_{EC} values were calculated as
149 described by Dictor, *et al.* (1998) to convert the ^{14}C-flush to ^{14}C-biomass based on the
150 original method of Sparling, *et al.* (1990).
151 To quantify the recovery of ^{14}C-activity, a mass balance was performed. The "un-
152 extractable" ^{14}C-residues in the soil pellets obtained after K_2SO_4 extraction of either
153 unfumigated or fumigated samples were quantified by LSC after sample oxidation as
154 previously described. The percentage recovery in relation to the added ^{14}C-glucose was
155 then calculated from the mass balance.
156
157 *2.3. Enumeration of bacterial populations in amended soils*
158 Culturable heterotrophic and hydrocarbon-degrading bacteria were enumerated as
159 colony forming unit (CFUs g^{-1}) following standard microbiological techniques.
160 Enumeration was carried out before and immediately after the 120-h radiorespirometric
161 assays; a portion of soil (2g) was mixed with 18 ml Ringer's solution and agitated on a

sonicator (20 min at 100 rpm). Samples used for cell enumeration after mineralisation were taken from the pellets obtained after the unfumigated soil portions were centrifuged. Aliquot of the soil suspension (1 ml) was ten-fold serially diluted, and 0.1 ml of the appropriate diluents inoculated on plate count agar (PCA), and agar plates impregnated with either phenanthrene or octacosane as the sole C-source. The plates were incubated in the dark at 25 °C and counted after 48 h for heterotrophic bacteria and approximately 8–10 d for phenanthrene- and octacosane-degrading bacteria.

2.4. Data analysis

Analysis of variance (ANOVA) was used to evaluate the effect of increasing diesel concentrations, and paired *t*-test was used to compare microbial ^{14}C-uptakes based on a fixed and the variable k_{EC} values. Data analysis was performed using IBM SPSS Statistics 19 for Windows (IBM SPSS Inc. Chicago, IL, USA). Statistical significance was set at 95% confidence level.

3. Results

3.1. Metabolic response of the extant soil microflora to fresh input of ^{14}C-glucose

The indices of mineralisation including lag phase, initial fastest rate and overall extent, indicated that ^{14}C-glucose mineralisation was dependent on diesel concentration and contact time in soil (Table 1). There were no effects on the lag phases and fastest rates at low diesel concentrations (1–100 mg kg^{-1}), but these indices were significantly affected ($P < 0.05$) at higher diesel concentrations (1000–10,000 mg kg^{-1}) across all soil–diesel contact times. This resulted in longer lag phases and reduced fastest rates as diesel concentration increased in soil. Extents of ^{14}C-glucose mineralisation decreased with increase in diesel concentration in soil, this being significant ($P < 0.05$) in the

187 1000 mg kg^{-1} treatment up to 42 d contact time and in the 10,000 mg kg^{-1} treatment up
188 to 63 d contact time.
189 After 1 d soil-diesel contact time and the 120-h radiorespirometric assays, about 38–
190 45% of the added ^{14}C-glucose was incorporated as ^{14}C-biomass in the control and
191 amended soils without a defined concentration-gradient (Table 1). After 21 and 42 d
192 contact times, ^{14}C-biomass was significantly greater ($P < 0.05$) in the 100–10,000 mg
193 kg^{-1} treatments than in the control. However, by 63 d contact time, ^{14}C-biomass was
194 higher only in the 10,000 mg kg^{-1} treatment relative to the control. The mass balances
195 indicated high recovery of the added ^{14}C-activity which was unaffected by diesel
196 concentration (Table 2). In general, total microbial ^{14}C-uptakes (i.e. $^{14}CO_2$ + ^{14}C-
197 biomass) decreased with increasing diesel concentration, and differed based on the k_{EC}
198 values used; the use of variable k_{EC} values gave a better account for the added ^{14}C-
199 actvity (Table 2).
200
201 *3.2. Microbial metabolic efficiency*
202 The biophysical quotient (BQ) and microbial yield coefficient (*Yc*) can be used to
203 evaluate the metabolic efficiency of the soil microbial community (Bradley & Fyles,
204 1995, Shen & Bartha, 1996).
205 The BQ was calculated as $\sum^{14}CO_2/^{14}$C-biomass.
206 The *Yc* was calculated as $\sum^{14}CO_2/(\sum^{14}CO_2 + ^{14}$C-biomass).
207 After 1 d contact time, the BQ fluctuated but with no clear concentration-gradient trend
208 (Figure 1). At 21 and 42 d contact times, the BQ decreased with increasing diesel
209 concentration in the 10–10,000 mg kg^{-1} treatments. After 63 d contact time, whilst the
210 values of the BQ were not markedly different ($P > 0.05$) in the control and 1–1000 mg
211 kg^{-1} treatments, it was significantly lower ($P < 0.05$) in 10,000 mg kg^{-1} treatment.

212 Similar to the trends observed for the BQ, the *Yc* showed no effect of diesel
213 concentration at 1 d soil–diesel contact time, whereas after 21 and 42 d contact times,
214 the *Yc* was significantly higher ($P < 0.05$) in the 10–10,000 mg kg^{-1} treatments
215 compared to the control soil. After 63 d contact time, while the *Yc* had become
216 comparable ($P > 0.05$) in control soil to the 1–1000 mg kg^{-1} treatments, it was still
217 significantly higher ($P < 0.05$) in 10,000 mg kg^{-1} treatment.
218
219 *3.3. Bacterial counts before and after ^{14}C-glucose mineralisation*
220 At each sampling time before the radiorespirometric assays, the CFUs of heterotrophic
221 bacteria were generally comparable ($P > 0.05$) in the control and amended soils (Figure
222 2A). The CFUs of phenanthrene- and octacosane-degrading bacteria were to some
223 extent higher in the 1000–10,000 mg kg^{-1} treatments than in the control, throughout the
224 duration of incubation (Figure 2A). In general, heterotrophic and hydrocarbon-
225 degrading bacterial cell numbers increased in all amended soils after compared to
226 before ^{14}C-radiorespirometric assays, at any of the sampling times, this being relatively
227 greater for hydrocarbon-degrading bacteria in the 100–10,000 mg kg^{-1} treatments
228 (Figure 2B).
229
230 **Discussion**
231 In this study, the measurements of ^{14}C-glucose mineralisation and ^{14}C-biomass
232 formation indicated that low diesel concentrations (1–100 mg kg^{-1}) have negligible
233 effects, whereas higher concentrations (1000–10,000 mg kg^{-1}) have significant effects
234 on the metabolic responses of the extant soil microflora of a pasture soil to fresh input
235 of ^{14}C-glucose. Remarkably, and perhaps a reflection of the temporal changes in the
236 abundance and physiological status and/or structure of the extant microbial community

237 in the 1000–10,000 mg kg^{-1} treatments, there was a significant shift in the pattern of
238 ^{14}C-glucose utilisation to greater allocation to ^{14}C-biomass; this was complemented by
239 the higher increases in the populations of hydrocarbon-degrading bacteria. Consistent
240 with the finding of this present study, the proportion of hydrocarbon-degrading bacteria
241 to the total heterotrophic populations has been observed to increase in soils following
242 exposure to coal tar or diesel, and in a landfill soil (Peña, *et al.*, 2007, Lors, *et al.*,
243 2010, Lors, *et al.*, 2012, Taccari, *et al.*, 2012). This suggests that the populations of
244 sensitive microbial species declined while those of tolerant and/or degrader species
245 increased in the amended soils. Concentration-dependent as well as time-dependent
246 successional changes in the physiological status and structural/functional diversity of
247 microbial communities in soils polluted with creosote and petroleum hydrocarbons
248 have been demonstrated (Phillips, *et al.*, 2000, Leys, *et al.*, 2005, Viñas, *et al.*, 2005,
249 Yrjälä, *et al.*, 2010, Lors, *et al.*, 2012, Kaczorek, *et al.*, 2013).
250 The introduction of relatively large amounts of labile carbon substrate, such as glucose,
251 to soils is able to shift the composition of soil microbial community from
252 autochthonous (humus-degrading) to zymogenous (opportunistic) microorganisms,
253 which are characterised by low metabolic efficiency, high turnover rates and low levels
254 of carbon incorporation into their biomass (Shen & Bartha, 1996). The increased
255 availability of ^{14}C-glucose to microorganisms has been shown to cause greater
256 mineralisation with corresponding lower allocation into ^{14}C-biomass (Shen & Bartha,
257 1996, Nguyen & Guckert, 2001). In this study, the pattern of microbial utilisation of
258 the added ^{14}C-glucose in the control is consistent with those described previously
259 (Bremer & Kuikman, 1994, Shen & Bartha, 1996, Nguyen & Guckert, 2001).
260 Collectively, the results of the BQ and *Yc*, as well as the relative changes in abundance
261 of hydrocarbon-degrading bacteria indicate that the extant soil microorganisms in the

262 soils amended with higher diesel concentrations have adapted to higher metabolic
263 efficiency by optimising their energy use through the diversion of more C derived from
264 glucose-C to biomass synthesis rather than to maintenance requirements. This kind of
265 energy optimisation strategy (i.e. improved C and energy utilisation) is typical of the
266 soil microbial community evolving a larger proportion of *k*-selected microorganisms
267 with higher metabolic efficiency and greater potential to utilise diverse and complex
268 substrates (Langer, *et al.*, 2004). Hence, it is hypothesised that the actively-growing
269 hydrocarbon-degrading and/or hydrocarbon-tolerant microorganisms in soils polluted
270 with high concentrations of petroleum hydrocarbons are more likely to preferentially
271 metabolise fresh input of readily-available and easier-to-degrade carbon substrates for
272 biosynthesis purposes rather than for respiratory purposes.
273 This finding underscores the role of endogenous or exogenously-supplied carbon
274 substrates to support biodegradation in petroleum hydrocarbon-polluted soils. In a
275 recent review of plant–bacteria partnerships for remediation of hydrocarbon-
276 contaminated soils, it was evidenced that labile carbon substrates from decaying plant
277 and root materials or living root exudates can promote the proliferation of
278 hydrocarbon-degrading microorganisms, resulting in enhanced biodegradation of
279 hydrocarbons in the rhizosphere (Khan, *et al.*, 2013). Several studies have reported
280 higher rates of pollutant removal during bioremediation of polluted soils and linked
281 these to the increased growth of hydrocarbon-degrading microorganisms stimulated by
282 the addition of readily-degradable carbon-containing materials (Liebeg & Cutright,
283 1999, Xu & Lu, 2012, Lladó, *et al.*, 2013, Pelaez, *et al.*, 2013).
284

Conclusions

In this study, it was demonstrated that the pattern of microbial utilisation of fresh input of labile carbon substrate like glucose is dependent on the initial contaminant level and contact time in diesel-impacted soil. The finding underscores the importance of considering the initial contaminant level when deciding on the use of labile carbon substrates to promote microbial proliferation during bioremediation.

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420

421

Tables

Table 1: Effect of increasing diesel concentration on microbial utilisation of ^{14}C-glucose, presented as the lag phase (h) initial fastest rate (% h^{-1}) and extent (%) of mineralisation, as well as biomass-^{14}C formation over 1–63 d contact times.

Time (d)	Concentration (mg kg^{-1})	Lag phase (h)	Init. fastest rate (% h^{-1}); T_{max} (h)	Extent (%)	^{14}C-Biomass (%)
1	0	2.49 ± 0.04[§a†]	2.12 ± 0.07[a] (<2)	52.67 ± 3.18[a]	42.52 ± 2.89[a]
	1	2.65 ± 0.27[a]	2.13 ± 0.17[a] (<2)	49.15 ± 1.86[a]	45.35 ± 1.54[a]
	10	2.44 ± 0.01[a]	2.08 ± 0.01[a] (<2)	51.20 ± 1.99[a]	41.58 ± 1.87[a]
	100	3.14 ± 0.70[a]	1.80 ± 0.38[a] (<2)	48.63 ± 0.67[a]	45.00 ± 2.77[a]
	1000	3.75 ± 0.35[b]	1.55 ± 0.05[b] (<2)	48.54 ± 1.62[a]	45.01 ± 1.54[a]
	10,000	3.59 ± 0.02[b]	1.42 ± 0.01[b] (<2)	54.37 ± 2.06[a]	38.16 ± 2.21[a]
21	0	2.53 ± 0.14[a]	2.15 ± 0.12[a] (<2)	51.85 ± 3.42[a]	43.34 ± 3.37[a]
	1	2.01 ± 0.31[a]	2.64 ± 0.38[a] (<2)	51.69 ± 1.70[a]	41.72 ± 2.41[a]
	10	2.34 ± 0.22[a]	2.23 ± 0.20[a] (<2)	47.47 ± 1.52[a]	47.90 ± 1.25[a]
	100	2.05 ± 0.27[a]	2.60 ± 0.22[a] (<2)	46.92 ± 0.68[a]	47.73 ± 1.89[a]
	1000	4.20 ± 0.36[b]	1.57 ± 0.05[b] (<2)	38.18 ± 1.24[b]	55.29 ± 1.45[b]
	10,000	4.69 ± 0.54[b]	1.45 ± 0.02[b] (<2)	33.16 ± 1.24[c]	55.23 ± 1.52[b]
42	0	2.57 ± 0.24[a]	2.03 ± 0.23[a] (<2)	49.58 ± 1.84[a]	45.19 ± 1.51[a]
	1	2.16 ± 0.06[a]	2.33 ± 0.06[a] (<2)	49.19 ± 1.17[a]	45.85 ± 1.28[a]
	10	2.36 ± 0.09[a]	2.18 ± 0.07[a] (<2)	50.56 ± 2.22[a]	43.63 ± 1.22[a]
	100	3.31 ± 0.76[b]	1.68 ± 0.36[a] (<2)	43.32 ± 2.58[ab]	53.06 ± 1.58[b]
	1000	5.96 ± 0.36[c]	0.90 ± 0.14[b] (2–4)	39.49 ± 2.77[b]	51.59 ± 3.12[b]
	10,000	5.08 ± 0.18[c]	1.07 ± 0.11[b] (2–4)	37.28 ± 1.65[c]	50.12 ± 1.44[b]
63	0	2.50 ± 0.02[a]	2.14 ± 0.06[a] (<2)	49.85 ± 2.23[a]	47.17 ± 2.30[a]
	1	2.09 ± 0.02[a]	2.42 ± 0.02[a] (<2)	46.58 ± 2.18[a]	48.87 ± 1.29[a]
	10	2.69 ± 0.30[a]	1.99 ± 0.22[a] (<2)	46.56 ± 0.56[a]	49.28 ± 0.50[a]
	100	2.20 ± 0.05[a]	2.35 ± 0.04[a] (<2)	46.47 ± 3.02[a]	49.51 ± 2.75[a]
	1000	8.05 ± 0.58[b]	0.83 ± 0.09[b] (2–4)	48.45 ± 3.67[a]	44.43 ± 2.37[b]
	10,000	10.46 ± 0.70[c]	0.73 ± 0.04[b] (4–6)	27.00 ± 1.07[b]	58.24 ± 0.87[c]

[§] Values indicate means of three replicates ± standard errors;
[†] Different lower-case letters indicate means that are statistically different ($P < 0.05$).

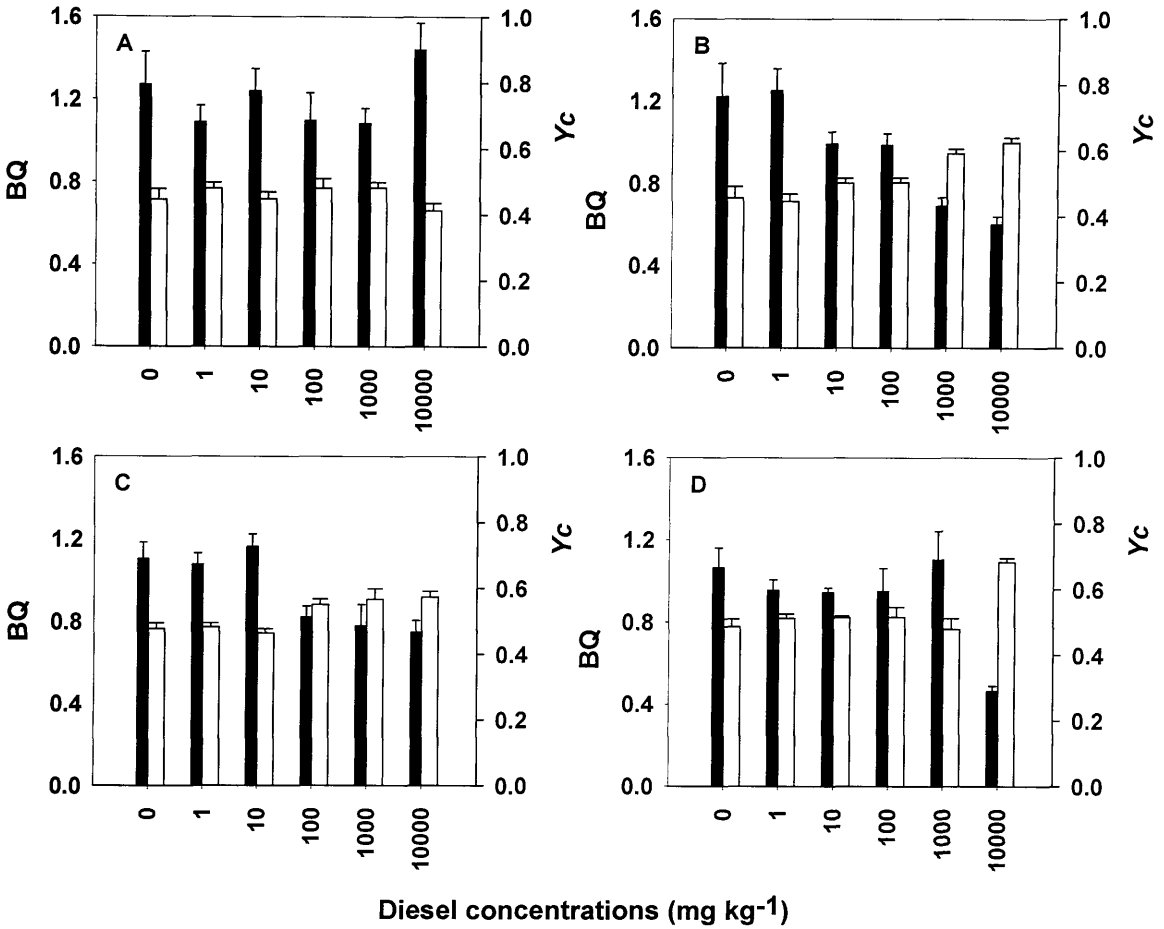
428 Table 2: Percentages of added ^{14}C-glucose ($^{14}C_{init}$) accounted for as $^{14}CO_2$ and ^{14}C-
429 biomass (i.e. microbial ^{14}C-uptake) using either fixed k_{EC} value (0.35) or variable k_{EC}
430 values and total ^{14}C-activity recovered.

Concentration (mg kg^{-1})	Microbial ^{14}C-uptake accounted for (%)		Total recovery (%)
	$^{14}CO_2$ + ^{14}C-Biomass (fixed)‡	$^{14}CO_2$ + ^{14}C-Biomass (variable)†	
0	91.31 ± 2.78^{a}	95.54 ± 1.28^{a}	101.94 ± 2.78
1	89.08 ± 1. 49^{a}	94.60 ± 0.32^{a}	103.07 ± 3.25
10	87.53 ± 3.62^{a}	94.54 ± 2.36^{a}	101.82 ± 2.20
100	74.09 ± 3.82^{b§}	95.16 ± 0.43^{a}	99.57 ± 2.73
1000	74.42 ± 2.88^{b§}	92.43 ± 0.52^{ab}	99.30 ± 2.51
10,000	60.51 ± 2.27^{c§}	88.17 ± 1.86^{b}	99.33 ± 2.93

431 ‡ Biomass-^{14}C based on a fixed k_{EC} value of 0.35
432 † Biomass-^{14}C based on derived k_{EC} values.
433 § Microbial ^{14}C-uptakes accounted for based on a fixed (0.35) and variable k_{EC} values are statistically different in
434 the amended soils ($P < 0.05$).
435

436 **Figures**

437



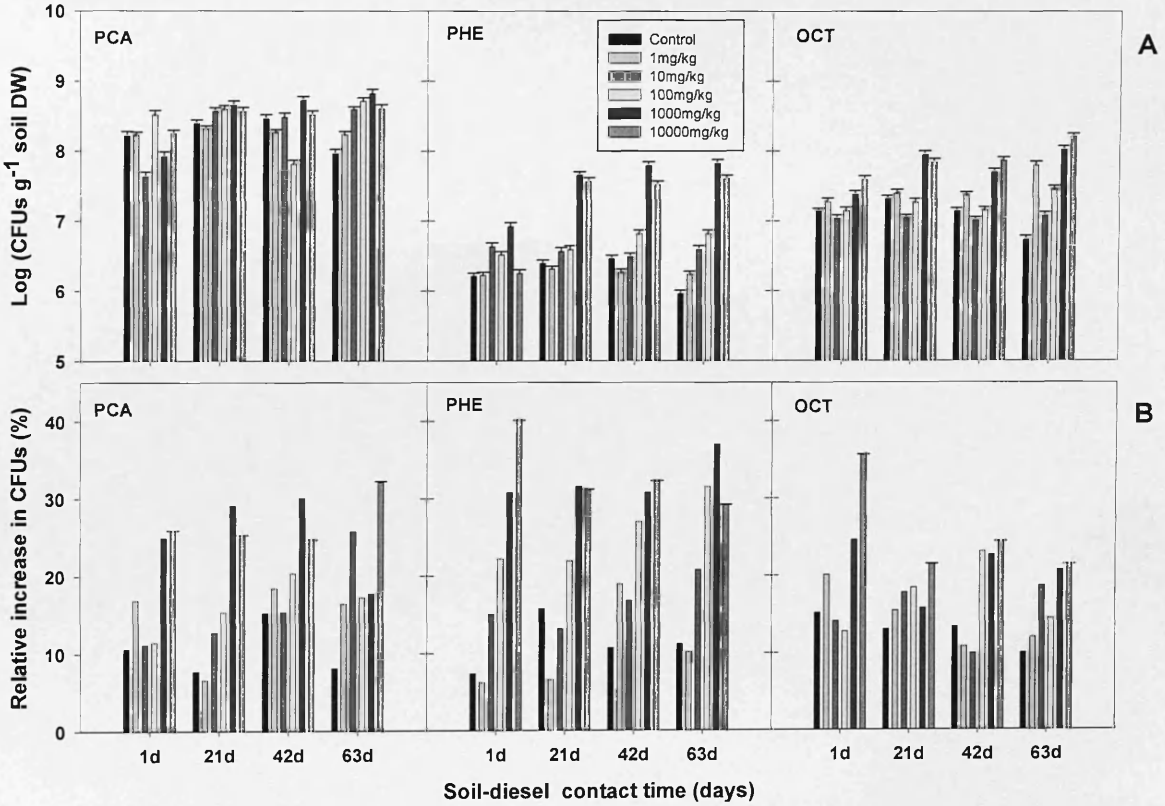
438
439

440 Figure 1: Effect of increasing diesel concentration on biophysical quotient (BQ) and

441 microbial yield coefficient (*Yc*) after (A) 1 (B) 21 (C) 42 and (D) 63 d contact times.

442

443



444
445

446 Figure 2: (A) effect of increasing diesel concentration on culturable heterotrophic,
447 phenanthrene and octacosane-degrading bacteria in soil before radiorespirometric
448 assays and (B) the relative increase after 120-h radiorespirometric assays. Data are
449 presented as means (n = 3) and error bars are the standard errors of mean (SEM).

450

Paper V

Biodegradation of phenanthrene and benzo[a]pyrene under complex contaminant systems in soils

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12 **Abstract**

13 Under complex hydrocarbon-contaminant mixtures systems, PAH catabolic activity
14 can be enhanced or repressed depending on contaminant concentration and contact
15 time in soil. However, the effects are rarely compared in different soil types. In this
16 study, the effect of diesel concentrations (0, 0.05 and 0.5% w/w) on the development of
17 phenanthrene and benzo[a]pyrene catabolism was compared in four different soils. The
18 indigenous catabolism of ^{14}C-PAHs was measured in freshly-amended and 4-week–
19 acclimated soils during 30-d soil slurry respirometric incubations. Results indicated
20 that due to the high intrinsic degradative potentials for phenanthrene in all the soils, the
21 presence and concentrations of diesel had minimal effect on the development of
22 phenanthrene catabolism. However, the presence of diesel and the soil–diesel contact
23 time were critical for benzo[a]pyrene catabolism in most soils. These findings
24 emphasise that more than for phenanthrene, the development of benzo[a]pyrene
25 catabolism varies widely with soil type, and depends on the concentration of co-
26 substrates present.

27

28 **Capsule:**

29 More than for phenanthrene, the development of benzo[a]pyrene catabolism varies
30 widely with soil type, and depends on diesel concentration and soil contact time.

31

32 **Keywords**: *Benzo[a]pyrene; Catabolic activity; Diesel oil; Microbial adaptation;*
33 *PAHs; Phenanthrene; Soil*

34

35 **1. Introduction**

36 Polycyclic aromatic hydrocarbons (PAHs) consist of a group of organic compounds
37 with two or more fused aromatic rings. PAHs are often present in contaminated
38 environments as constituents of heterogeneous non-aqueous phase liquids (NAPLs),
39 such as creosote and coal tars, as well as crude and refined petroleum oils (Kanaly and
40 Watanabe, 2004). PAH contamination of soil presents serious environmental and health
41 concerns because of their persistence; this is due, in part, to low water solubility, low
42 volatility, high lipophilicity, and the recalcitrant molecular structure (Hu *et al.*, 2012).
43 Biodegradation is widely viewed as the principal mechanism of PAH removal from
44 soils and is affected predominantly by the properties of the contaminant that influence
45 its bioavailability and chemical recalcitrance, environmental and edaphic factors, such
46 as soil physicochemical characteristics, as well as catabolic potential of indigenous
47 microbial populations (Couling *et al.*, 2010). Another important factor that may impact
48 on PAH biodegradation, in field contaminated soil, is the presence of other
49 hydrophobic organic contaminants (HOCs), in that it is very rare to find a situation
50 where contamination arises from the presence of a single chemical. Biodegradation of a
51 PAH may proceed at different rates and to different extents due to various biological,
52 chemical and/or physical limitations or changes induced by the presence of co-
53 contaminants (Ghoshal *et al.*, 1996). These changes may affect the level of extant
54 microbial activity as well as extent of bioavailability and the subsequent
55 biodegradation of the PAH.

56 Evidence from previous studies, suggests that that the development of PAH catabolism
57 is enhanced or repressed in the presence of other HOCs depending on a number of
58 factors (Bauer and Capone, 1988; Efroymson and Alexander, 1994; Labare and
59 Alexander, 1995; Kanaly *et al.*, 1997; Kanaly *et al.*, 2001). Such factors include the

type and concentration of the co-contaminant, the kinds of microorganisms present and their catabolic preferences. For example, the catabolism of PAHs containing four and five aromatic rings has been shown to be enhanced by the presence of other PAHs, containing fewer rings (Bauer and Capone, 1988). This was suggested to be as a result of the microbial populations having either broad specificity for PAHs, common catabolic pathways, or both. The composition and concentration of co-contaminants are also thought to affect the development of PAH catabolic activity (Kanaly *et al.*, 1997; Kanaly *et al.*, 2001; Couling *et al.*, 2010). The authors demonstrated that the antagonistic and/or synergistic interactions between microbial populations as well as between multiple contaminants may affect the fate of PAHs in soil. Most of these studies, however, have been conducted using binary or tertiary mixtures of hydrocarbons, with only a few conducted in soils containing complex mixtures of hydrocarbons, which reflect environmental scenarios and are more representative of soils polluted with diesel, coal tar or creosote. Moreover, a direct quantitative comparison is generally lacking which considers variability in soil type and the physicochemical properties of target contaminants.

Therefore, this study investigated the effect of diesel concentration on the development of indigenous catabolism of a 3-ring PAH, phenanthrene, and a 5-ring PAH, benzo[a]pyrene, in four different soils.

2. Materials and methods

2.1. Chemicals

Unlabelled PAHs and [9-^{14}C]phenanthrene (55.7 mCi mmol^{-1}; >99% purity) were supplied by Sigma–Aldrich (Poole, UK) and [7-^{14}C]benzo[a]pyrene (13.8 mCi mmol^{-1}; >95% purity) was from Amersham Corp. (Arlington Heights, Ill). Goldstar

multipurpose liquid scintillation fluid and 7-ml glass scintillation vials were supplied by Meridian (Epsdom, UK) while sodium hydroxide was from Merck (UK). Diesel oil used in this experiment was a commercial grade no. 2 fuel obtained at a local BP fuel station in Lancaster, UK. The oil had total organic carbon content of 87%, and contained 86% diesel range organics (nC$_8$–nC$_{25}$) and 10% gasoline range organics (nC$_6$–nC$_{10}$), as determined by gas chromatography coupled with flame ionisation detection (GC-FID). Phenanthrene but not benzo[a]pyrene was detected. All other solvents and chemicals used were of reagent grade or better.

2.2. Soils sampling and bulk characterisation

The Antarctic soil was obtained from Livingstone Island, the Antarctica; while the other three soils were collected from various sites at Nether-Kellet, Holme and Thurnham in the UK (see Table 1). At the time of sampling, the Nether-Kellet soil was under grassland, while the Holme and Thurnham soils were prepared for the winter cropping season. Except for the Antarctic soil, the soils were collected fresh from the field, air-dried for ~48 h to about 40% of their water holding capacity (WHC) and passed through 2-mm sieve to remove large roots and stones. The physicochemical properties of soils are presented in Table 1. Particle size analysis was determined according to the method by Gee and Bauder (1979) and calculations according to Gee and Bauder (1986). Soil pH and moisture content were measured by standard methods described elsewhere (Rhodes *et al.*, 2007). For the determination of soil organic carbon (SOC) total nitrogen (TN) contents, soils were pre-treated using the procedure of Nieuwenhuize *et al.* (1994) as adapted by Rhodes *et al.* (2007). A Carlo Erba CHNS-OEA 1108 CN-Elemental Analyser was used for quantification.

109 The microbiological indices of the soils were determined prior to the start of this study.
110 Microbial respiratory activity (basal and substrate-induced), respiratory quotient (RQ:
111 ratio of CO_2 production to O_2 consumption) and biomass-C, as well as metabolic
112 quotient (qCO_2: CO_2/biomass-C), microbial quotient (biomass-C–to–SOC) of soils
113 were quantified by measuring the respiration rates in an aerobic closed static system on
114 a MicroOxymax respirometer (Multiple Sensor O_2/CO_2 10-Chamber System,
115 Columbus). The procedure for the set-up and operation of the respirometer has been
116 described elsewhere (Towell *et al.*, 2011). Fungal–to–bacterial biomass-C ratio was
117 estimated using the substrate-induced respiration (SIR) method (Anderson and
118 Domsch, 1978) combined with selective inhibition techniques (Nakamoto and
119 Wakahara, 2004). Oxytetracycline hydrochloride (4 mg g^{-1} DW soil) and
120 cycloheximide (2 mg g^{-1} DW soil) were used as bacterial and fungal inhibitors,
121 respectively. Culturable heterotrophic and PAH-degrading bacterial cells were
122 enumerated by the standard spread plate method (Lorch *et al.*, 1995).
123 The background concentrations of total petroleum (aromatic and aliphatic)
124 hydrocarbons (TPH) were quantified by shake-extraction of samples in 1:1 solution of
125 dichloromethane/acetone for 24 h. Extracts were passed through alumina column to
126 clean up and separate the aliphatic and aromatic components. Quantitative analysis was
127 performed with (GC-FID), essentially following the EPA Method 8015 (US EPA,
128 1987).
129
130 *2.3. Soil preparation and microcosm setup*
131 Soils were amended with diesel at concentrations of 0, 500 and 5000 $mg_{oil\text{-}C}\ kg^{-1}{}_{soil}$
132 (hereafter referred to as unamended, 0.05%-amended and 0.5%-amended soils,
133 respectively) using the one-step spiking/rehydration procedure as previously described

by Doick *et al.*, 2003. Soil samples (200 g) were placed in pre-cleaned amber jars covered with perforated aluminium foil and then acclimatised in the dark at controlled temperature (22.0 ± 1 °C) and humidity (45%) for 30 d. Where necessary, the moisture contents of the amended soils were re-adjusted to 60% WHC in order to maintain microbial viability throughout the incubation period.

2.4. Mineralisation of ^{14}C-PAHs

Soil (10 ± 0.2 g) was placed in 250-ml standard respirometer bottles with 30 ml sterilise minimum basal salts (MBS) solution. Either phenanthrene (incorporated as 50 mg ^{12}C-phenanthrene kg^{-1}_{soil} with associated ^{14}C-activity *ca.* 83 Bq g^{-1}_{soil}) or benzo[a]pyrene (incorporated as 10 mg ^{12}C-benzo[a]pyrene kg^{-1}_{soil} with associated ^{14}C-activity of ~83 Bq g^{-1}_{soil}) was added to the respirometers (Reid *et al.*, 2001). Toluene (5 µl per respirometer) was used as carrier solvent for the hydrocarbons because benzo[a]pyrene did not readily dissolved in acetone. The recommended soil:liquid ratio of 1:3 was used to allow for optimal biodegradation of the accessible ^{14}C-PAH fractions (Doick and Semple, 2003). The sealed respirometers were incubated at 21 ± 1 °C and shaken at 100 rpm on a bench–top orbital shaker (Janke and Kunkel, IKA®–Labortechnik KS250) for thorough mixing of the contaminants in the soil slurry. The $^{14}CO_2$ evolved from the catabolism of the ^{14}C-PAHs was trapped in suspended vials containing 1 M NaOH (1 ml) and periodically quantified by liquid scintillation counting (LSC, Canberra Packard Tri Carb 2300TR, UK). Phenanthrene catabolism was monitored for 18 d because its mineralisation usually plateaued at about 8–12 d while benzo[a]pyrene was monitored for 30 d because of its extended lag phase. All treatment set-ups were in triplicate. Blank respirometers were also set-up to monitor background radioactivity in the soils. All respirometry data, initially corrected for

159 background radioactivity and machine noise, were used to calculate the overall extents
160 (%), maximum rates (% d^{-1}) and T_{max} (d; time taken to reach the maximum rate), as
161 well as lag phase (d; time taken for cumulative mineralisation to exceeds 5% of added
162 ^{14}C-PAH).
163
164 *2.5. Enumeration of PAH-degrading microorganisms before and after mineralisation*
165 The numbers of phenanthrene and benzo[a]pyrene degraders in the soils before and
166 after ^{14}C-PAH mineralisation were quantified as colony-forming units (CFUs) on agar
167 plates impregnated with either phenanthrene or benzo[a]pyrene as sole source of
168 carbon and energy following standard microbiological procedures (Lorch *et al.*, 1995).
169
170 *2.6. Statistical analysis*
171 The graphing of mineralisation data was undertaken with SigmaPlot v12.0 (SPSS Inc.,
172 Chicago, IL). All statistical analyses were performed using SigmaStat v3.05 (SPSS
173 Inc.) following the satisfaction of normality tests where necessary. Comparisons for
174 statistical significance were made between diesel concentrations and soil types for the
175 various mineralisation indices. Holm-Sidak method was used for all pairwise multiple
176 comparison. Statistical significance was set at $P \leq 0.05$.
177
178 **3. Results**
179 *3.1. Soil physicochemical and microbiological properties*
180 Physicochemical and microbiological properties of soils are presented in Table 1.
181 Particle size distributions showed variation between the soils; the Holme soil had the
182 largest silt and clay fractions (89.55%), while the Antarctic soil was predominantly
183 comprised of sand (94.69%). SOC and N contents varied significantly ($P < 0.05$) with

184 highest values (19.50% and 1.32%) and lowest values (0.25% and 0.28%) in the Holme
185 and the Antarctic soils, respectively. Moisture content varied considerably, ranging
186 from 5.62 to 48.44% and pH ranged from 5.44 to 7.50.
187 Microbial respiratory responses and biomass-C sizes differed ($P < 0.05$) between the
188 soils (Table 1). The Antarctic soil had the lowest values for both basal and substrate-
189 induced respiration (0.11 and 5.84 μg CO_2-C g^{-1} h^{-1}, respectively), as well as biomass-C
190 (102.98 μg-C g^{-1} soil). Basal and SIR respiration were highest (9.62 and 144.98 μg
191 CO_2-C g^{-1} h^{-1}, respectively) in the Thurnham soil, while biomass-C was highest in the
192 Holme soil (2343.81 μg-C g^{-1}). Ratios of fungal–to–bacterial biomass-C (0.02–1.25)
193 and biomass-C–to–SOC (0.012–0.059) differed between the soils. Meanwhile, the
194 respiratory quotients (RQs; basal: 0.73–0.91 and SIR: 1.02–1.25 μg CO_2-C μg^{-1} O_2)
195 were relatively similar. Heterotrophic CFUs ranged between 1.2×10^5 and 4.9×10^8 in
196 the Antarctic and the Nether-Kellet soils, respectively. For both phenanthrene- and
197 benzo[a]pyrene-degrading bacteria, CFUs were lowest in the Antarctic soil and highest
198 in the Thurnham soil. Overall, there were no correlations between any of the abiotic
199 characteristic of soil and the measured biotic parameters.
200 There was no known record of exposure to petroleum hydrocarbons in any of the soils.
201 The background levels of ∑PAHs (9.98–114.81 μg kg^{-1}) and aliphatic hydrocarbons
202 (2.16–115.36 μg kg^{-1}), consisting mainly of C_{17}–C_{30} chain length compounds, varied
203 significantly ($P < 0.05$) between soils (Table 2). However, background ∑PAHs
204 concentrations were generally low and negligible compared to the amounts of diesel
205 spiked into the soils. The levels of ∑PAHs in the UK soils are about 5–12 times higher
206 than in the Antarctic soil, and are generally poorly correlated with SOC or soil particle
207 sizes ($r^2 < 0.32$; $P > 0.05$). Overall, weak correlations ($r^2 < 0.43$; $P > 0.05$) were found
208 between the background levels of ∑PAHs, phenanthrene or benzo[a]pyrene and the

measured biotic parameters, such as the size and overall activity (e.g., qCO_2, RQ) of microbial biomass or CFUs of bacteria able to utilise the PAHs.

3.2. Mineralisation of ^{14}C-phenanthrene in diesel-amended soils

Mineralisation of ^{14}C-phenanthrene was monitored over a period of 18 d in freshly-amended and 4-week–acclimated soils (Figure 1; Table 2). The catabolic activity for phenanthrene was generally high in all of the soils; indicated by short lag phases (3–8 d), high maximum rates (6–19% d^{-1}), and elevated extents (50–66%) of ^{14}C-phenanthrene mineralisation (Table 2). While maximum rates and extents of mineralisation were comparable in the Nether-Kellet, Holme and Thurnham soils, the lag phases and T_{max} were significantly shorter in Thurnham soil, in which there was the highest phenanthrene degrading CFUs. The Antarctic soil, containing the lowest background phenanthrene concentration and numbers of phenanthrene degraders, also exhibited the lowest levels of indigenous catabolic activity, as shown by the longest lag phase, the slowest maximum rates and lowest extents of mineralisation of ^{14}C-phenanthrene. Statistically, strong correlations were found between the populations of phenanthrene degraders and the lag phase (r^2 = 0.97; P = 0.01) and between background levels of phenanthrene in soils and the maximum rates (r^2 = 0.89; P = 0.05). However, neither of these correlated with the cumulative extents of mineralisation; further, none of the abiotic properties of soil correlated with any of the indices of mineralisation.

The effect of diesel concentration on ^{14}C-phenanthrene mineralisation in freshly-amended soils differed between the soils. For example, there were no significant effects ($P > 0.05$) on mineralisation in the Nether-Kellet or Thurnham soils. However, extents of ^{14}C-phenanthrene mineralisation were significantly higher ($P < 0.05$) in both of the

234 0.05%-amended Antarctic and Holme soils, but significantly lower ($P < 0.05$) in the
235 0.5%-amended Holme soil, compared to their respective unamended soils (Figure 1;
236 Table 2). The microbial and physicochemical characteristics of the Antarctica and
237 Holme soils are quite different (Tables 1 and 3). Furthermore, relatively longer lag
238 phases and slower maximum rates (with longer T_{max} values) of ^{14}C-phenanthrene
239 mineralisation were observed in all 0.5%-amended soil samples compared to their
240 0.05%-amended counterparts; though usually statistically insignificant ($P > 0.05$).
241 Pre-exposure generally promoted microbial degradation, but its effect on the
242 development of phenanthrene catabolic activity was dependent on both soil type and
243 diesel concentration. For example, after 4 weeks soil–diesel contact time, the lag
244 phases were significantly ($P < 0.05$) shorter, but extents of ^{14}C-phenanthrene
245 mineralisation remained similar in all of the diesel amended samples of the Antarctic
246 and Nether-Kellet soils and the 0.5%-amended Thurnham soil. However, phenanthrene
247 catabolism was appreciably enhanced, with significantly ($P < 0.05$) shorter lag phases
248 and higher extents of ^{14}C-phenanthrene mineralisation, in both the amended samples of
249 the Holme soil and the 0.05%-amended Thurnham soil (Table 2). Moreover, when
250 compared with the freshly-amended soil samples, the maximum rates of ^{14}C-
251 phenanthrene mineralisation in all 4-week–acclimated soil samples were either similar
252 or slightly higher, but the T_{max} values were considerably shorter.
253 Overall, addition of fresh 0.05% diesel to the 4-week–acclimated soils apparently had
254 negligible effects on the soil microflora to mineralise ^{14}C-phenanthrene further (Table
255 2). However, the presence of fresh diesel had variable influence on the different aspects
256 of phenanthrene degradation endpoints depending on the soil type and initial diesel
257 concentration. For example, the lag phases of ^{14}C-phenanthrene mineralisation were
258 shorter after additional amendment of most 4-week–acclimated soil samples; being

259 significant in the 0.5%-amended Antarctic soil, both of the amended samples of
260 Nether-Kellet soil and the 0.05%-amended Holme and Thurnham soils. Generally, T_{max}
261 values were shorter in all soil samples that received fresh diesel, but the effects on
262 maximum rates were variable in the soils depending on the initial diesel concentration.
263 For example, in the Nether-Kellet soil, after additional amendment with diesel, the
264 maximum rates were significantly slower in 0.05%-amended samples, while
265 significantly faster in the 0.5%-amended samples. Except in the 0.05%-amended
266 Nether-Kellet soil, additional amendment only marginally increased the extents of ^{14}C-
267 phenanthrene mineralisation in all of the 4-week–acclimated soil samples.
268
269 *3.3. Mineralisation of ^{14}C-benzo[a]pyrene in diesel-amended soils*
270 Mineralisation ^{14}C-benzo[a]pyrene was monitored over a period of 30 d in freshly-
271 amended and 4-week–acclimated soils (Figure 2; Table 3). The intrinsic catabolic
272 activity for benzo[a]pyrene was generally low in all soils, except for the Nether-Kellet
273 soil (Table 3). The mineralisation indices indicated relatively long lag phases (13–>30
274 d), slow maximum rates (0.03–1.32% d^{-1}), and variable extents (<1–28%) of ^{14}C-
275 benzo[a]pyrene mineralisation.
276 Overall, no aspects of benzo[a]pyrene degradation endpoints statistically correlated
277 with any of the measured biotic parameters. For instance, the biomass content and its
278 activity, as well as the numbers of heterotrophic and benzo[a]pyrene-degrading
279 bacteria were higher in the Holme than in the Antarctic soil, but benzo[a]pyrene
280 catabolic activity was lower in the Holme soil. Also, CFUs of benzo[a]pyrene
281 degraders in the Nether-Kellet and Thurnham soils were comparable (Table 1), but ^{14}C-
282 benzo[a]pyrene mineralisation in these soils differed significantly ($P < 0.05$) (Table 3).
283 The presence of benzo[a]pyrene at background levels in the Nether-Kellet and

284 Thurnham soils appeared to have influence on the ability of soil microflora to
285 mineralise the ^{14}C-PAH. Further, statistically there were no strong correlations, but it
286 seemed that physicochemical properties of the soil influenced the bioaccessibility of
287 benzo[a]pyrene in that lag phases tended to increase and maximum rates and extents of
288 mineralisation decreased as SOC, silt and clay contents increased in the soils.
289 In the freshly-amended soils, the development of benzo[a]pyrene catabolism depended
290 on the interactions between soil type and diesel concentration. In comparison to the
291 unamended soils, the lag phases were significantly shorter ($P < 0.05$) in both amended
292 samples of the Antarctic soil, the extent of ^{14}C-benzo[a]pyrene mineralisation was
293 significantly higher (~4-fold) in 0.05%-amended soil only. Furthermore, whilst the
294 extents of ^{14}C-benzo[a]pyrene mineralisation increased significantly ($P < 0.05$) in both
295 amended samples of the Nether-Kellet soil, the lag phases were comparable to the
296 unamended soils. However, benzo[a]pyrene catabolism was enhanced with
297 significantly ($P < 0.05$) shorter lag phases and higher extents of ^{14}C-benzo[a]pyrene
298 mineralisation in all amended samples of the Holme and Thurnham soils. It is
299 noteworthy that whilst enhancement of benzo[a]pyrene catabolism was modest (*ca.* 2-
300 fold) in the freshly-amended Nether-Kellet soils, with the highest inherent catabolic
301 potential, it was much greater in the freshly-amended Holme (>10-fold) and Thurnham
302 soils (>6-fold), despite having lower indigenous catabolic activity. The diesel
303 concentration had different effects on benzo[a]pyrene mineralisation indices. For
304 example, the extent of ^{14}C-benzo[a]pyrene mineralisation was significantly higher in
305 the 0.05%-amended Antarctic soil, but significant lower in 0.05%-amended Thurnham
306 soil, as compared to the respective 0.5%-amended soils. Furthermore, relatively longer
307 lag phases and slower maximum rates, with longer T_{max} values were observed in all of
308 the 0.5%-amended soil samples, compared to the 0.05%-amended counterparts; though

309 usually statistically insignificant ($P > 0.05$). Similar effects on ^{14}C-phenanthrene were
310 observed in the freshly-amended soils.
311 Pre-exposure generally promoted microbial adaptation and significantly enhanced ($P <$
312 0.05) the development of benzo[a]pyrene catabolic activity in all soils and at both
313 diesel concentrations (Figure 2; Table 3). For example, after 4 weeks soil–diesel
314 contact, there were significant increases ($P < 0.05$) in ^{14}C-benzo[a]pyrene
315 mineralisation in all of the diesel-amended Nether-Kellet, Holme and Thurnham soils;
316 indicated by shorter lag phases, faster maximum rates with shorter T_{max} values, and
317 greater extents of mineralisation. In both of the amended samples of the Antarctic soil,
318 the lag phases were slightly shorter, but there were significant ($P < 0.05$) increases in
319 maximum rates and extents of ^{14}C-benzo[a]pyrene mineralisation. With respect to
320 diesel concentration, significantly ($P < 0.05$) shorter lag phases and greater extents of
321 ^{14}C-benzo[a]pyrene mineralisation were obtained in the 0.05%-amended Antarctic soil,
322 compared to 0.5%-amendment. The opposite was observed in the diesel amended
323 samples of the Holme and Thurnham soils.
324 Overall, addition of fresh 0.05% diesel to the 4-week–acclimated soils further
325 enhanced the capacity of all of the soils to mineralise ^{14}C-benzo[a]pyrene (Table 3).
326 Additional amendment with diesel resulted in significantly greater extents ($P < 0.05$) of
327 ^{14}C-benzo[a]pyrene mineralisation in all of the diesel-amended Antarctic and Holme
328 soils, as well as the 0.05%-amended Nether-Kellet and Thurnham soils, compared to
329 the respective unamended soils. Also, the lag phases in some of the diesel amended
330 soils were significantly shorter, while maximum rates were faster after additional diesel
331 amendment. However, additional amendment had no effect on ^{14}C-benzo[a]pyrene
332 mineralisation indices in 0.5%-amended samples of the Nether-Kellet and Thurnham
333 soils. Furthermore, additional amendment appeared to have variable effects on T_{max};

these being shorter in most 0.05%-amended and longer in some 0.5%-amended soil samples.

3.4. Microbial numbers before and after mineralisation of ^{14}C-PAHs in diesel-amended soils

The CFUs of PAH-degrading bacteria in the freshly-amended and 4-week–acclimated soils are presented in Table 4. The CFUs of phenanthrene-degrading bacteria increased following 4-week acclimation of all of the amended soils; being generally higher in the higher diesel concentration. After ^{14}C-phenanthrene mineralisation, the number of phenanthrene degraders significantly increased in all of the soils, irrespective of diesel concentration, indicating that the biodegradation process supported proliferation of the degrading cells. Addition of fresh diesel appeared to promote the growth of phenanthrene degraders further with significantly higher ($P < 0.05$) CFUs in most of the soils. The CFUs of benzo[a]pyrene-degrading bacteria were significantly lower (about 2 to 4 orders of magnitude) in all of the soils than that of the phenanthrene-degrading bacteria. After 4 weeks of acclimation, the CFUs of benzo[a]pyrene degraders generally increased (by approximately 1 to 2 orders of magnitude) in all the diesel amended soils. After ^{14}C-benzo[a]pyrene mineralisation, the CFUs of benzo[a]pyrene degraders further increased beyond that before mineralisation. Addition of fresh diesel appeared to promote the growth of benzo[a]pyrene degraders further, with slightly higher CFUs in most of the soils.

356 **4. Discussion**

357 *4.1 Relationship between background ∑PAH concentrations and soil properties*

358 The relative abundance of some individual PAHs, such as naphthalene, phenanthrene
359 and perylene to the ∑PAH concentrations indicates a greater contribution from natural
360 than anthropogenic sources to all the soils investigated (Wilcke, 2007). In the UK soils,
361 naphthalene, phenanthrene, anthracene, and benzo[*k*]fluoranthene are the dominant
362 PAHs, and their background ∑PAH levels are comparable to values (<600 μg
363 ∑16PAHs kg^{-1}) obtained in other soils from rural UK (Jones *et al.*, 1989). The levels of
364 individual PAHs and ∑PAHs in the Antarctic soil are comparable with values reported
365 in the literature for soils from the region and other remote sites (Wilcke, 2000; Okere
366 and Semple, 2012). In this study, the background ∑PAH and aliphatic hydrocarbon
367 concentrations appeared not to have been influenced by SOC or the particle size
368 distribution; though, strong correlations between background ∑PAH levels and the
369 holding capacity of soils (i.e. sink-related factors) had been reported (Agarwal *et al.*,
370 2009; Nam *et al.*, 2009). This may be as a result of differences in the regional
371 locations, paths of deposition and exposure histories of the soils investigated. The
372 significantly higher background levels of ∑PAHs, especially the HMW-PAHs, found
373 in the UK soils than in the Antarctic soil can be attributed to closer proximity of the
374 UK soils to long-term anthropogenic input sources and the long history of
375 industrialisation in the UK (Johnsen and Karlson, 2005; Nam *et al.*, 2009). Soils from
376 remote sites, such as the Antarctic, are thought to be predominantly influenced by long-
377 range atmospheric transport and to a less degree by deposition from local sources (Nam
378 *et al.*, 2009).

379 There are active heterotrophic communities which include sizeable populations of
380 PAH-degrading microorganisms in the soils. The total as well as relative abundance of

381 fungal–to–bacterial biomass contents and the respiratory activities in these soils are
382 comparable with soils under similar land use or from similar regions (Lin and Brookes,
383 1999; Aislabie *et al.*, 2001). Quotients, such as RQ, qCO_2, and biomass-C–to–SOC, are
384 widely used to indicate the current eco-physiological status and energetic maintenance
385 requirement of soil microbial communities and their metabolic efficiency (Wardle and
386 Ghani, 1995; Dilly *et al.*, 2001). Overall, these biotic indicators did not reflect the
387 background ∑PAH and aliphatic hydrocarbon concentrations in these soils. However,
388 results suggest that despite the large differences in the SOC and microbial
389 communities, the quality of the mineralisable portions of SOC and microbial O_2
390 demand for C oxidation are comparable in the soils investigated (Dilly *et al.*, 2001).
391
392 *4.2 Phenanthrene mineralisation in unamended and amended soils*
393 As previously observed by Rhodes *et al.* (2010) and Okere *et al.* (2012), phenanthrene
394 catabolism is inherently high in the soils. The results are in agreement with the finding
395 of Johnsen and Karlson (2005), who attributed the presence of large populations of
396 microorganisms able to mineralise phenanthrene in uncontaminated soils due to the
397 ubiquitous nature of the PAH in the environment, even at very low concentrations.
398 Extents of phenanthrene mineralisation are comparable in all soils despite the high
399 variability in soil texture and SOC. This is consistent with the finding of Yang *et al.*
400 (2009), who demonstrated that SOC and porosity properties, such as soil surface area,
401 had a compounding effect on microbial availability of phenanthrene in soils. In that
402 study, the authors observed that though the mineralisation of freely dissolved
403 phenanthrene by the bacterium PYR-1 was initially faster in soils with lower SOC, the
404 extents of mineralisation only varied by a factor of 1.6–2.1 after 9 d for all five soils
405 with SOC ranging from 0.23 to 7.1%. The results of this present study highlight that

406 even in soils with varying degrees of catabolic potentials extent of phenanthrene
407 mineralisation may not differ much as a result of soil-dependent bioavailability
408 limitations.
409 The high levels of indigenous phenanthrene catabolic activity in the soils most likely
410 ensured that the presence of diesel as co-contaminants, at the range of concentrations
411 (500–5000 mg kg^{-1}) investigated, had marginal effects on the extents of phenanthrene
412 catabolism. This is consistent with the findings of Swindell and Reid (2006), who
413 showed that the presence of diesel, at concentrations up to and including 2000 mg kg^{-1},
414 had negligible effects on the different aspects of ^{14}C-phenanthrene mineralisation in
415 soil; whereas, at concentrations of 10,000–20,000 mg kg^{-1} significantly longer lag
416 phases and lower rates and extents of mineralisation were observed (Swindell and
417 Reid, 2006; Figure 4). This was attributed, in part, to increased toxicity at higher diesel
418 concentration and, in part, to greater competitive inhibitory effect by certain more
419 really biodegradable components of diesel (Bossert and Bartha, 1986). The observation
420 that acclimation to diesel improved the ability of indigenous soil microflora to
421 mineralise phenanthrene is consistent with the findings of other studies that have
422 investigated the development of HOC catabolism in soil (Spain *et al.*, 1980; Macleod
423 and Semple, 2002; Swindell and Reid, 2006; Rhodes *et al.*, 2008). An implication of
424 the results is that although the development of phenanthrene catabolic activity might be
425 transiently repressed in the presence of high concentrations of co-contaminants,
426 extensive degradation and mineralisation of phenanthrene as source of carbon and
427 energy will certainly occur in soils contaminated with diesel up to 5000 mg kg^{-1}.
428 Further, the results of this study indicated that diesel may not necessarily serve as co-
429 substrate for phenanthrene biodegradation. This is probably because most soil

430 microflora can readily initiate phenanthrene degradation without prior cometabolic
431 induction of their enzyme systems (Seo *et al.*, 2009).
432
433 *4.3 Benzo[a]pyrene mineralisation in unamended and amended soils*
434 The low extents of ^{14}C-benzo[a]pyrene mineralisation recorded in most of the
435 background soils investigated was not surprising in that benzo[a]pyrene is not readily
436 accessible and is highly recalcitrant to microbial oxidation (Seo *et al.*, 2009).
437 Mineralisation of ^{14}C-benzo[a]pyrene is thought to be limited by the lack of suitable
438 co-substrates for cometabolism as well as the low abundance of degrader populations
439 and/or inadequate assemblages of competent degrading consortia in the soils (Kanaly *et*
440 *al.*, 2002). The relatively low levels of benzo[a]pyrene can also limit the development
441 of catabolic ability in background soils (Seo *et al.*, 2009). Hydroxypropyl-β-
442 cyclodextrin (HP-β-CD) extraction indicated that 71, 21, 13 and 20% of the added ^{14}C-
443 benzo[a]pyrene (10 mg kg^{-1}) was bioaccessible (i.e. the potentially biodegradable
444 fraction) in Antarctic, Nether-Kellet, Holme and Thurnham soils, respectively.
445 Collectively, the results show that the mineralisation of benzo[a]pyrene in soil was
446 largely limited by either low intrinsic catabolic potential or absence of suitable co-
447 substrates for cometabolic degradation, or both, and to a lesser extent by
448 bioaccessibility, and that the effect of these factors differed with soil types. Thus, the
449 effect of soil abiotic properties to limit the bioavailability of benzo[a]pyrene seems to
450 be negligible and secondary to the effect of soil biotic characteristics on mineralisation,
451 as other authors have also reported (Roper and Pfaender, 2001; Derz *et al.*, 2006).
452 Several investigators have reported that enhanced or repressed mineralisation of
453 benzo[a]pyrene is dependent on the complex interrelationships between a variety of
454 factors, such as concentration and nature of co-substrates, kinds of organisms and their

455 growth conditions as well as bioaccessibility limitations (Chen and Aitken, 1999;
456 Kanaly and Bartha, 1999; Kanaly *et al.*, 2001; Bogan *et al.*, 2003; Kanaly and
457 Watanabe, 2004; Zang *et al.*, 2007). For instance, the results presented here are in
458 agreement with those of Kanaly and Watanabe (2004) who observed that at sufficient
459 concentrations, high-boiling point distillate components of diesel supported extensive
460 mineralisation of benzo[a]pyrene by an enriched bacterial mixed culture consisting of
461 genera, such as *Sphingomonas*, *Mycobacterium* and *Burkholderia*. In that study, diesel
462 was thought to have acted as co-solvent for benzo[a]pyrene dissolution and/or as
463 inducers of co-metabolism as well as promoting the growth of catabolically-competent
464 microbial populations in soil, though the precise mechanisms were not elucidated
465 The findings of this study are particularly remarkable with respect to the Antarctic and
466 Nether-Kellet soils. Based on the current literature, this is the first time extensive
467 benzo[a]pyrene mineralisation is reported in an Antarctic soil, naturally or artificially
468 polluted. To date, mineralisation of mainly *n*-alkanes and two- to four-ringed PAHs has
469 been reported in soils from this region (Baraniecki *et al.*, 2002; Aislabie *et al.*, 2012;
470 Okere *et al.*, 2012). Studies of biodegradation of hydrocarbons in Antarctic soils have
471 been reviewed (Aislabie *et al.*, 2004; Aislabie *et al.*, 2006). The results indicated that
472 given the right conditions, as obtainable in *ex-situ* bioreactor settings, wherein
473 microbial growth can be optimised and maintained, bioaugmentation with indigenous
474 soil microflora from this soil can cause rapid and extensive mineralisation of
475 benzo[a]pyrene. The relevance to the detoxification of contaminated sites in the
476 Antarctic is that since the Protocol on Environmental Protection to the Antarctic Treaty
477 (1991; see Article 4 and Appendices B & C of Annex II to the Protocol) imposes
478 stringent restrictions on Party States to import extraneous microorganisms to the
479 region, native soil microflora, if enriched, can be successfully used to degrade HMW

480 PAHs. So far only a few of the studies of benzo[a]pyrene biodegradation by indigenous
481 soil microflora in pristine environments have reported measurable mineralisation
482 (>5%), most studies reported non to marginal mineralisation (Grosser *et al.*, 1991;
483 Carmichael and Pfaender, 1997; Kanaly *et al.*, 2006). To the authors' knowledge, this
484 is also the first study to demonstrate extensive (>20%) benzo[a]pyrene mineralisation
485 by indigenous soil microflora in a pristine soil from the temperate region. Overall, the
486 findings of this study further emphasised the ubiquitous nature of microbial catabolic
487 ability and the potentials within microbial communities to adapt to degrade any
488 xenobiotic compound.
489
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493

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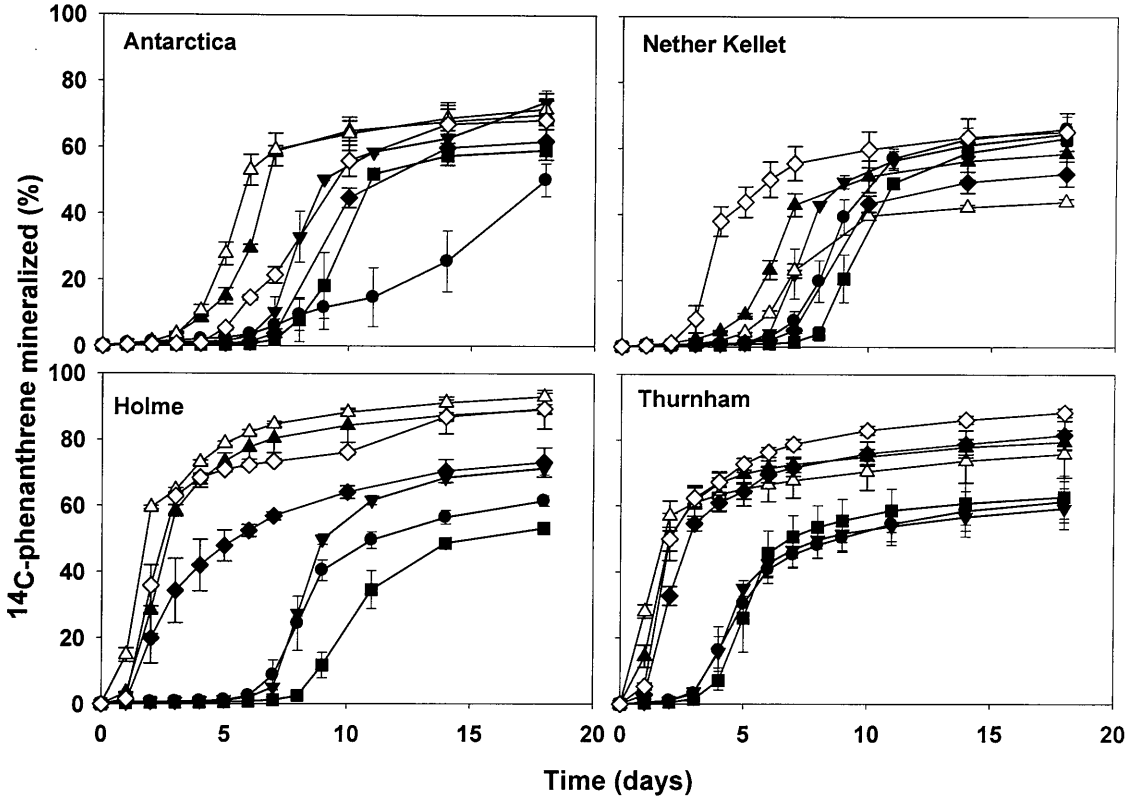
651

652 **Figures**

653



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655

656 Figure 1: Catabolism of phenanthrene (50 mg kg^{-1}_{soil}) in unamended soils (●), freshly-
657 amended soils amended with diesel at 500 $mg_{oil\text{-}C}$ kg^{-1}_{soil} (▼) or at 5000 $mg_{oil\text{-}C}$ kg^{-1}_{soil}
658 (■) and 4-week–acclimated soils amended with diesel at 500 $mg_{oil\text{-}C}$ kg^{-1}_{soil} (▲) with
659 additional fresh diesel (Δ) or at 5000 $mg_{oil\text{-}C}$ kg^{-1}_{soil} (♦) with additional fresh diesel (◊).
660 The concentration of fresh diesel added was 5000 $mg_{oil\text{-}C}$ kg^{-1}_{soil}. Error bars, where
661 visible, are 1 SEM (n = 3).

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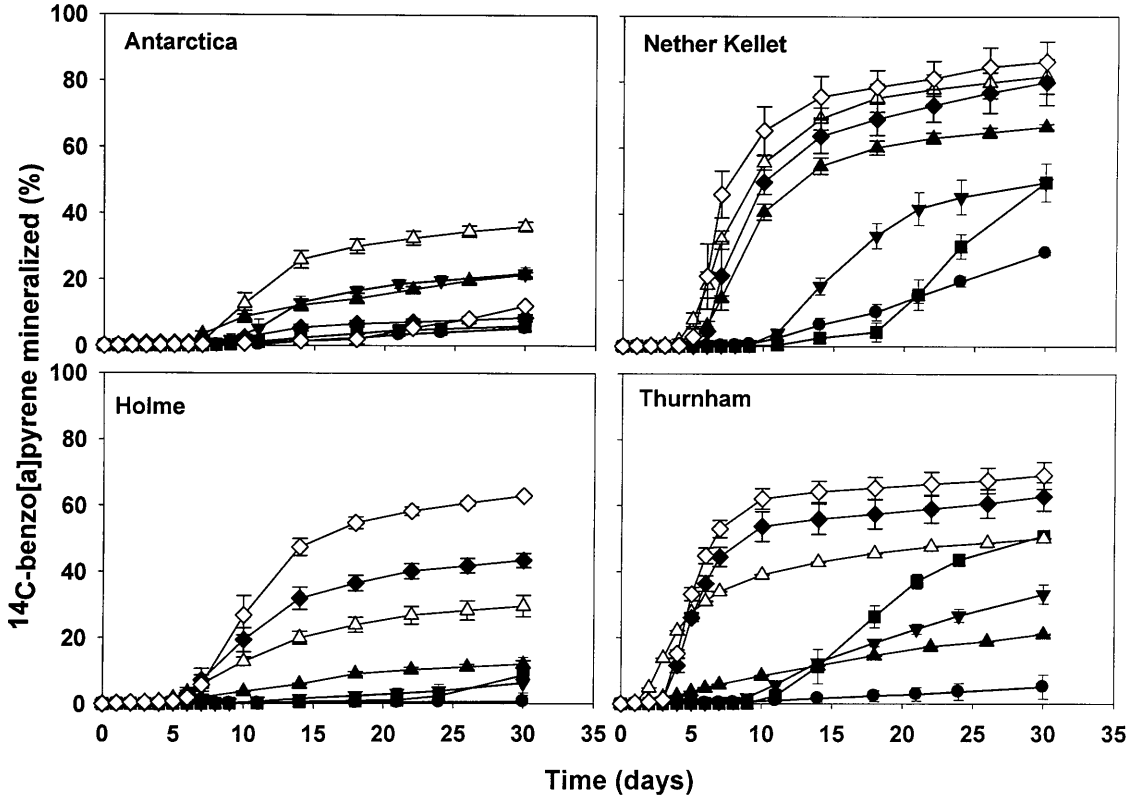

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667 Figure 2: Catabolism of benzo[a]pyrene (10 mg kg^{-1}_{soil}) in unamended soils (●),
668 freshly-amended soils amended with diesel at 500 $mg_{oil\text{-}C}$ kg^{-1}_{soil} (▼) or at 5000 $mg_{oil\text{-}C}$
669 kg^{-1}_{soil} (■) and 4-week–acclimated soils amended with diesel at 500 $mg_{oil\text{-}C}$ kg^{-1}_{soil} (▲)
670 with additional fresh diesel (Δ) or at 5000 $mg_{oil\text{-}C}$ kg^{-1}_{soil} (♦) with additional fresh diesel
671 (◊). The concentration of fresh diesel added was 5000 $mg_{oil\text{-}C}$ kg^{-1}_{soil}. Error bars, where
672 visible, are 1 SEM (n = 3).

673 **Tables**

674 Table 1: Soil physicochemical properties and selected microbiological indicators.

Soil Characteristics		Antarctic	Nether-Kellet	Holme	Thurnham
Soil classification		Typical sandy	Typical brown-earth	Earthy oligo-fibrous peat	Typical humic alluvial gley
Grid reference		Antarctica	SD491655	SD511775	SD447543
Texture		Sandy	Loam	Silty clay	Clay loam
Particle size analysis	Sand Course	-	7.53	0.96	0.92
(%)	Medium	91.59	11.17	1.80	1.20
	Fine	3.10	36.69	7.69	36.65
	Total sand	94.69	55.39	10.45	38.77
	Silt	5.31	26.77	47.37	34.44
	Clay	0	17.84	42.18	27.79
pH	dH_2O	6.25	5.44	7.50	6.93
	$CaCl_2$	-	5.02	6.01	5.19
Moisture content (%)		5.62±0.36	22.61±0.90	48.44±0.85	34.21±0.63
Total organic carbon (SOC; %)		0.25	2.99±4.71	19.50±6.20	3.48±5.10
Total nitrogen (TN; %)		0.28	0.25±0.31	1.32±0.42	0.26±0.52
Respiratory responses	Basal†	0.11±0.05	7.11±0.15	9.05±0.20	9.62±0.65
($\mu g\ CO_2$-C $g^{-1}\ h^{-1}$)	SIR‡	5.84±0.71	72.03±1.25	99.11±2.84	144.98±2.12
Biomass-C ($\mu g\ g^{-1}$) #		102.98±12.45	1361.57±106.49	2343.81±44.04	2040.42±49.89
Fungal/Bacterial ratio		0.02	1.09	1.02	1.25
Biomass/SOC (%)		0.041	0.046	0.012	0.059
qCO_2 ($\mu g\ mg^{-1}$ biomass h^{-1})		1.07	5.22	3.86	4.72
RQ (mol CO_2 mol^{-1} O_2)¶	Basal	0.84±0.10	0.73±0.01	0.89±0.04	0.91±0.03
	SIR	1.10±0.14	1.15±0.01	1.02±0.00	1.25±0.01
Microbial numbers ($CFU\ g^{-1}$)‡	Heterotrophic	1.16 (0.41) x 10^5	4.90 (1.04) x 10^8	2.59 (0.67) x 10^8	3.07 (1.23) x 10^8
	Phenanthrene	3.20 (1.12) x 10^3	1.70 (2.75) x 10^7	1.35 (0.50) x 10^7	4.10 (1.45) x 10^7
	Benzo[a]pyrene	2.10 (1.10) x 10^2	5.20 (1.10) x 10^3	1.30 (0.80) x 10^3	6.20 (1.50) x 10^3

675 qCO_2: Metabolic quotient
676 RQ: Respiratory quotient
677 † Values (mean ± SEM) are average of 2-hourly continuously measurements for 24 h.
678 ǂ SIR is the maximum glucose-induced respiration within 24 h.
679 # Determined by SIR method
680 ¶ Values (mean ± SEM) are average of 2-hourly continuously measurements for 24 h in triplicate samples.
681 ‡ Values in parentheses are SD of 4 replicate counts.
682

683 Table 2: Distribution of aliphatic and polycyclic aromatic hydrocarbons in soils.

Hydrocarbons (μg kg^{-1} DW soil)	Antarctic	Nether-Kellet	Holme	Thurnham
PAHs				
Naphthalene	ND	30.91	6.53	ND
Acenaphthylene	ND	0.64	1.43	ND
Acenaphthene	ND	0.76	1.55	ND
Fluorene	1.16	1.55	1.37	0.77
Phenanthrene	1.13	18.90	34.01	19.30
Anthracene	0.66	15.21	16.86	9.94
Fluoranthene	ND	6.66	1.44	0.39
Pyrene	0.69	2.50	1.72	2.28
Benzo[a]anthracene	1.47	3.34	2.21	ND
Chrysene	0.32	0.66	1.06	0.56
Benzo[*b*]fluoranthene	1.29	3.60	3.53	0.44
Benzo[*j*]fluoranthene	1.05	3.36	1.74	1.61
Benzo[*k*]fluoranthene	0.62	10.55	11.21	5.94
Benzo[*a*]pyrene	ND	1.41	ND	0.42
Perylene	1.07	4.38	5.20	3.96
Indeno[1,2,3-*cd*]pyrene	0.53	4.92	7.97	1.13
Dibenz[*ah*]anthracene	ND	1.64	0.76	0.72
Benzo[*ghi*]perylene	ND	ND	ND	0.40
Coronene	ND	3.85	6.15	1.97
∑19PAHs	9.98	114.81	104.71	49.81
Aliphatic hydrocarbons				
C_8–C_{12}	ND	ND	ND	ND
C_{13}–C_{20}	0.50	0.06	1.63	1.29
Phytane	ND	ND	4.69	2.88
C_{21}–C_{29}	11.48	2.02	101.13	31.74
C_{30}–C_{35}	0.64	0.08	12.60	2.82
∑C_8–C_{35}	12.62	2.16	115.36	35.85

684 ND: Below detection limit

685 Table 3: Effect of diesel concentration on ^{14}C-phenanthrene mineralisation in freshly-

686 amended and 4-week–acclimated soils.

Soil type	Time	Concentration, ($mg_{oil\text{-}C}$ kg^{-1}_{soil})	Lag phase, (d)	Maximum rate, (% d^{-1}); T_{max} (d)	Overall extent, $\sum^{14}CO_2$ (%)	Impact factor
Antarctic	Unamended	0	7.84±1.63 [a]§	6.15±2.67 [a] (8–18)†	50.23±5.00 [a]	–
	Fresh	500	6.55±0.37 [a]	22.64±3.31 [c] (6–9)	73.78±3.52 [b]	1.47
	Fresh	5000	8.13±0.59 [a]	16.86±4.42 [b] (8–11)	59.23±1.12 [a]	1.18
	4-week	500 + 0	3.30±0.05 [b]	19.76±9.67 [b] (7)†	69.95±4.35 [b]	1.39
	4-week	500 + 500	3.25±0.13 [b]	24.69±4.35 [c] (6)	71.68±4.80 [b]	1.43
	4-week	5000 + 0	7.04±0.15 [a]	13.66±0.62 [b] (10)	61.91±5.58 [b]	1.23
	4-week	5000 + 500	5.96±0.84 [c*]	11.52±3.90 [b] (6–10)	68.37±5.92 [b]	1.36
Nether-Kellet	Unamended	0	6.64±0.33 [a]	19.48±2.99 [a] (8–9)	66.08±3.81 [a]	–
	Fresh	500	6.06±0.13 [a]	20.86±7.45 [a] (7–8)	64.59±3.33 [a]	0.98
	Fresh	5000	8.28±0.04 [e]	17.32±0.63 [a] (9–11)	63.25±2.31 [a]	0.96
	4-week	500 + 0	4.06±0.06 [c]	19.69±3.33 [a] (7)	58.60±6.19 [c]	0.88
	4-week	500 + 500	5.11±0.01 [d*]	5.73±4.65 [b*] (7)	43.97±0.93 [b*]	0.67
	4-week	5000 + 0	5.36±1.57 [d]	12.82±0.58 [c] (10)	52.49±3.80 [c]	0.79
	4-week	5000 + 500	2.75±0.27 [b*]	29.66±3.44 [d*] (4)	65.22±5.72 [a*]	0.99
Holme	Unamended	0	6.74±0.34 [a]	16.05±5.98 [a] (8–9)	61.42±1.58 [a]	–
	Fresh	500	6.90±0.13 [a]	22.46±1.95 [c] (8–9)	71.25 ±1.95 [c]	1.16
	Fresh	5000	6.76±1.62 [a]	11.43±0.98 [b] (9–11)	53.17±1.12 [b]	0.86
	4-week	500 + 0	1.08±0.03 [c]	30.15±8.87 [c] (2–3)	89.10±5.83 [d]	1.45
	4-week	500 + 500	0.35±0.05 [b*]	44.58±2.50 [d*] (2)	92.99±1.12 [d]	1.51
	4-week	5000 + 0	2.63±1.34 [d]	13.36±7.77 [a] (2–3)	73.08±4.41 [c]	1.19
	4-week	5000 + 500	1.87±0.76 [d]	23.26±11.71 [c] (2–3)	89.30±1.33 [d*]	1.45
Thurnham	Unamended	0	3.30±0.41 [a]	14.10±2.53 [a] (4–5)	61.39±6.28 [a]	–
	Fresh	500	3.15±0.11 [a]	19.39±7.13 [a] (4–5)	59.29±6.19 [a]	0.97
	Fresh	5000	3.71±0.25 [a]	19.63±4.60 [a] (5–6)	62.79±6.55 [a]	1.02
	4-week	500 + 0	0.40±0.12 [b]	36.21±3.92 [b] (2)	79.49±2.41 [b]	1.29
	4-week	500 + 500	0.18±0.01 [c*]	28.92±6.15 [b] (1–2)	75.86±7.20 [b]	1.24
	4-week	5000 + 0	1.07±0.01 [d]	29.89±2.88 [b] (2–3)	81.56±4.29 [b]	1.33
	4-week	5000 + 500	0.87±0.09 [d]	44.61±2.91 [c*] (2)	88.36±2.20 [b]	1.44

687 § Different lower-case letters down the column within each soil type indicate mean values of triplicate samples that are
688 significantly different from the control ($P < 0.05$).
689 † Values in parentheses are time taken for mineralisation to reach maximum rates in days ($n = 3$)
690 * Significant difference ($P < 0.05$) between 4-week–acclimated soils amended with or without additional fresh diesel
691

692 Table 4: Effect of diesel concentration on ^{14}C-benzo[a]pyrene mineralisation in freshl-

693 amended and 4-week–acclimated soils.

Soil type	Time	Concentration, ($mg_{oil\text{-}C}\ kg^{-1}_{soil}$)	Lag phase, (d)	Maximum rate, (% d^{-1}); T_{max} (d)	Overall extent, $\sum^{14}CO_2$ (%)	Impact factor
Antarctic	Unamended	0	28.74±0.65 [a]§	0.34±0.02 [a] (18–21)†	5.31±0.19 [a]	–
	Fresh	500	11.92±0.20 [c]	2.62±0.50 [c] (9–14)	21.85±1.81 [d]	4.11
	Fresh	5000	19.05±4.44 [c]	0.39±0.32 [a] (11–21)	5.93±1.44 [a]	1.12
	4-week	500 + 0	8.20±0.83 [d]	2.76±0.83 [c] (7)†	21.52±0.73 [d]	4.05
	4-week	500 + 500	8.13±0.33 [d]	3.79±1.07 [c] (10–14)	36.06±1.38 [e*]	6.79
	4-week	5000 + 0	14.68±2.39 [c]	0.74±0.08 [b] (10–14)	8.41±1.00 [b]	1.58
	4-week	5000 + 500	22.00±2.33 [b*]	0.18±0.17 [a*] (2–30)	11.98±0.17 [c*]	2.26
Nether-Kellet	Unamended	0	13.53±1.41 [a]	1.32±0.77 [a] (14–21)	28.49±0.83 [a]	–
	Fresh	500	11.04±0.28 [a]	4.73±1.06 [b] (14–18)	49.73±5.74 [b]	1.75
	Fresh	5000	16.72±2.66 [a]	0.71±0.44 [a] (21–24)	49.83±1.76 [b]	1.75
	4-week	500 + 0	6.04±0.33 [b]	8.06±1.05 [c] (7–10)	66.62±0.83 [c]	2.33
	4-week	500 + 500	4.63±0.26 [c*]	13.69±1.73 [d*] (6–7)	81.92±5.26 [d*]	2.86
	4-week	5000 + 0	6.67±0.48 [b]	13.00±10.26 [d] (7–10)	80.13±6.94 [d]	2.81
	4-week	5000 + 500	5.16±0.27 [c*]	24.80±6.35 [d] (6–7)	86.30±6.11 [d]	3.03
Holme	Unamended	0	>30 [a]	0.03±0.02 [a] (4–8)	0.61±0.21 [a]	–
	Fresh	500	30 [a]	0.26±0.14 [c] (11–30)	6.21±3.76 [b]	10.18
	Fresh	5000	30 [a]	0.12±0.02 [b] (30)	8.60±5.39 [b]	14.09
	4-week	500 + 0	12.27±0.24 [b]	0.77±0.15 [d] (10–18)	11.93±1.02 [c]	19.56
	4-week	500 + 500	6.66±0.28 [c*]	2.44±0.26 [e*] (7)	29.54±3.23 [d*]	48.43
	4-week	5000 + 0	6.73±0.60 [c]	4.11±1.72 [f] (7–10)	43.36±2.15 [e]	71.08
	4-week	5000 + 500	6.96±0.44 [c]	6.99±1.03 [g*] (10–14)	62.79±0.45 [f*]	102.93
Thurnham	Unamended	0	>18[#a]	0.22±0.17 [a] (24–30)	5.16±3.63 [a]	–
	Fresh	500	10.61±0.19 [b]	2.25±0.22 [b] (11–14)	33.26±2.88 [c]	6.45
	Fresh	5000	12.44±1.20 [b]	3.78±0.82 [c] (14–18)	50.94±1.43 [d]	9.87
	4-week	500 + 0	6.22±0.91 [c]	0.49±0.39 [a] (5)	21.09±0.09 [b]	4.09
	4-week	500 + 500	2.02±0.07 [e*]	3.04±2.96 [bc*] (3)	50.15±0.12 [d*]	9.72
	4-week	5000 + 0	3.41±0.12 [d]	14.50±1.79 [d] (4–5)	62.84±4.32 [e]	12.18
	4-week	5000 + 500	3.28±0.04 [d]	18.10±0.79 [e*] (5)	69.20±4.07 [e]	13.41

694 § Different lower-case letters down the column within each soil type indicate mean values of triplicate samples that are
695 significantly different from the control ($P < 0.05$).
696 † Values in parentheses are time taken for mineralisation to reach maximum rates in days ($n = 3$)
697 * Significant difference ($P < 0.05$) between 4-week–acclimated soils amended with or without additional fresh diesel
698 # Only one of the triplicate samples reached >5% mineralisation before the end of the experiment
699

700 Table 5: Indigenous bacterial degrader numbers (CFU g^{-1}) in freshly-amended and 4-
701 week–acclimated soils before and after mineralisation of ^{14}C-phenanthrene or ^{14}C-
702 benzo[a]pyrene; values are presented as the means ± standard error of the mean (n = 3).

Soil type	Time (d)	Concentration, ($mg_{oil\text{-}C}\ kg^{-1}{}_{soil}$)	Phenanthrene (x10^6 CFUs g^{-1})		Benzo[a]pyrene (x10^4 CFUs g^{-1})	
			Before	After	Before	After
Antarctic	Unamended	0	<0.01	5.40±0.67	<0.20	<0.5
	Fresh	500	<0.01	15.40±4.40	<0.20	1.75±0.72
	Fresh	5000	<0.01	11.60±1.10	<0.20	1.40±0.45
	4-week	500 + 0	3.12±0.88	11.40±3.20	2.87±1.10	11.40±3.50
	4-week	500 + 500	3.12±0.88	24.00±6.10	2.87±1.10	9.55±3.60
	4-week	5000 + 0	10.20±6.63	25.10±6.00	6.50±3.20	13.80±4.40
	4-week	5000 + 500	10.20±6.63	38.70±3.40	6.50±3.20	10.40±5.10
Nether-Kellet	Unamended	0	17.70±2.70	34.50±4.50	0.52±0.11	5.30±3.60
	Fresh	500	17.70±2.70	37.50±0.67	0.52±0.11	7.50±0.56
	Fresh	5000	17.70±2.70	49.50±4.30	0.52±0.11	10.70±6.70
	4-week	500 + 0	46.00±8.33	76.00±6.00	26.30±4.30	40.80±4.70
	4-week	500 + 500	46.00±8.33	91.00±10.50	26.30±4.30	44.50±4.50
	4-week	5000 + 0	47.00±7.70	90.50±7.50	30.40±8.30	56.10±6.10
	4-week	5000 + 500	47.00±7.70	108.00±13.50	30.40±8.30	60.40±3.40
Holme	Unamended	0	13.50±5.00	55.20±4.30	0.13±0.08	0.55±0.33
	Fresh	500	13.50±5.00	63.80±0.67	0.13±0.08	4.50±3.30
	Fresh	5000	13.50±5.00	55.60±0.33	0.13±0.08	4.50±5.10
	4-week	500 + 0	33.50±7.80	45.20±10.00	2.56±0.64	13.40±3.30
	4-week	500 + 500	33.50±7.80	62.60±8.30	2.56±0.64	15.40±4.50
	4-week	5000 + 0	34.60±6.50	49.60±13.10	7.45±3.30	28.41±5.60
	4-week	5000 + 500	34.60±6.50	66.30±4.50	7.45±3.30	30.40±5.20
Thurnham	Unamended	0	41.00±14.50	58.50±7.30	2.62±0.15	3.84±0.63
	Fresh	500	41.00±14.50	59.60±8.60	2.62±0.15	10.30±4.10
	Fresh	5000	41.00±14.50	49.50±12.10	2.62±0.15	12.50±0.56
	4-week	500 + 0	55.60±7.70	64.70±5.40	16.50±5.00	23.10±4.30
	4-week	500 + 500	55.60±7.70	71.50±8.30	16.50±5.00	31.20±0.76
	4-week	5000 + 0	56.00±8.35	60.10±16.10	10.50±1.50	40.13±3.30
	4-week	5000 + 500	56.00±8.35	83.10±12.70	10.50±1.50	41.00±10.30

703

Paper VI

Effects of HP-β-CD concentrations and repeated exposures to diesel on biodegradation of benzo[a]pyrene in soil

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Effects of HP-β-CD concentrations and repeated exposures to diesel on biodegradation of benzo[a]pyrene in soil

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11 **Abstract**

12 It is well-known that low aqueous solubility and poor microbial degradative potential
13 limit the removal of benzo[a]pyrene from soil. In this study, the effects of
14 hydroxypropyl-β-cyclodextrin (HP-β-CD) concentrations (0, 12.5, 25 and 50 mM) and
15 repeated exposures over 150 d to diesel oil (1x500, 1x5000, 2x250, 2x2500, 5x100 and
16 5x1000 $mg_{oil\text{-}C}$ kg^{-1}_{soil}) on benzo[a]pyrene mineralisation were assessed in soil.
17 Indigenous catabolic activity towards benzo[a]pyrene was low in soil (mineralisation
18 extent <1%). However, catabolic activity was significantly enhanced (by 11 to 55
19 times); this being greatest in soils repeatedly exposed to the lower diesel
20 concentrations. Overall, whilst the presence of 50 mM HP-β-CD significantly
21 increased the apparent aqueous dissolution of benzo[a]pyrene from soil matrices, it
22 failed to enhance benzo[a]pyrene mineralisation; however, lower HP-β-CD
23 concentrations appeared to have some impact on mineralisation. These findings are
24 important in assessing the fate of benzo[a]pyrene and in designing bespoke remediation
25 strategies for soils chronically exposed to petroleum-derived oils.

26

27 **Capsule**:

28 Increasing HP-β-CD concentration significantly increased the apparent aqueous
29 dissolution of B[a]P from soil matrices, but failed to further enhance ^{14}C-B[a]P
30 mineralisation.

31

32 **Keywords**: *Benzo[a]pyrene; Bioaccessibility; Biodegradation; Diesel oil;*
33 *Hydroxypropyl-β-cyclodextrin; Mineralisation*

34

Highlights:

- Pre-exposure to diesel significantly enhanced B[a]P catabolic activity in soil
- B[a]P mineralisation greatest after repeated exposures to lower diesel concentrations
- HP-β-CD significantly increased the apparent aqueous dissolution of B[a]P
- Increasing HP-β-CD concentrations had variable effects on B[a]P mineralisation.

40 **1. Introduction**

41 Soils and sediments polluted with polycyclic aromatic hydrocarbons (PAHs),
42 particularly those of higher-molecular-weights, such as benzo[a]pyrene (B[a]P), are of
43 major public health concern since these chemicals are toxic and exhibit carcinogenic
44 and mutagenic properties (Juhasz and Naidu 2000). The presence of B[a]P in
45 contaminated soils is especially problematic due to its high resonance energy,
46 extremely low aqueous solubility, low volatility, strong affinity for particulate matters
47 and highly recalcitrant chemical structure – properties which make the contaminant to
48 persist in the environment (Bamforth and Singleton 2005; Juhasz and Naidu 2000). In
49 addition, the ability of PAHs, and in particular B[a]P, to accumulate in living plant and
50 animal tissues and form extremely reactive bulky adducts with DNA and proteins are
51 particularly worrisome for human health (Vrabie et al., 2011). Comparatively, these
52 attributes make the B[a]P removal process more challenging than most of the other
53 PAHs often encountered in contaminated soils.

54 The limitations of low aqueous solubility and high affinity for soil particles can be
55 overcome by the addition of chemicals which can enhance the solubility of
56 hydrophobic organic contaminants in water, making the contaminants more accessible
57 for microbial uptake and degradation. Unlike most other solubility enhancement agents
58 like surfactants, cyclodextrins (CDs) are known to have minimal affinity to soil
59 matrices, negligible cytotoxic effects on soil biota, and are relatively biodegradable,
60 making their direct applications an important property for bioremediation (Molnar et
61 al., 2005; Wang et al., 1998). An example of a cyclodextrin that has been widely
62 studied for its ability to improve PAH solubility and biodegradation in both laboratory
63 and field studies is 2-hydroxypropyl-β-cyclodextrin (HP-β-CD). Several investigators
64 have reported significantly improved PAH degradation rates when supplemented with

65 HP-β-CD (Allan et al., 2007; Bardi et al., 2000; Berselli et al., 2004; Carroll and
66 Brusseau 2009; Wang et al., 2005; Wang et al., 1998). Few studies have reported no or
67 marginal effects (Cuypers et al., 2002; Rafin et al., 2009; Stroud et al., 2009; Ye et al.,
68 1996), and none, so far have reported negative effects on PAH biodegradation. Most of
69 the studies that reported on HP-β-CD-enhanced solubilisation and biodegradation of
70 PAHs were focussed mainly on lower molecular weight PAHs with relatively fewer
71 studies on PAHs with four or more benzene rings (e.g., Wang et al., 2005). Further,
72 previous studies have usually been carried out in liquid media with isolated pure or
73 mixed cultures whose degradative properties have been enhanced *in vivo* (Wang et al.,
74 2005; Ye et al., 1996).
75 B[a]P is the most widely-studied PAH in terms of ecotoxicity because it is known to
76 exhibit strong carcinogenic, mutagenic and immuno-toxigenic properties (Juhasz and
77 Naidu 2000). Comparatively, however, little effort has been given to investigate its
78 biodegradation in the environment (Seo et al., 2009). Apart from the intractable
79 physicochemical characteristics of B[a]P, the low susceptibility to microbial attack and
80 inability to serve as a C-source for most microorganisms makes B[a]P biodegradation a
81 major challenge in contaminated soils. Moreover, catabolic activity for B[a]P is very
82 low or even non-existent in contaminated soils (Kanaly and Harayama 2000). This has
83 been attributed to poor distribution of degradative traits in the gene pools within
84 microbial populations, which limits adaptation and development of degradative ability
85 in most soil microflora (Bamforth and Singleton 2005). Usually, when B[a]P
86 biodegradation occurs, it follows a long period of adaptation to analogous co-
87 contaminants (Juhasz and Naidu 2000). A number of studies have examined aspects of
88 co-contaminant parameters, such as composition and concentration, which promote or
89 repress biodegradation of B[a]P (Chen and Aitken 1999; Kanaly et al., 1997; Kanaly

90 and Harayama 2000). What remains poorly understood are the factors and mechanisms
91 that influence the development B[a]P degradative activity in contaminated soils. For
92 example, there is limited information on the effect of repeated exposures on the
93 development of B[a]P degradative activity in soils contaminated with complex co-
94 contaminant mixtures, such as diesel oil.
95 Therefore, this study has two main objectives: i) to assess the effect of multiple
96 exposures to diesel on the development of B[a]P degradative ability in soil, and ii) to
97 assess the effect of the presence and increasing concentration of HP-β-CD on
98 solubilisation and biodegradation of B[a]P in unexposed and exposed soils. To the
99 authors' knowledge, this is first report on HP-β-CD-enhanced mineralisation of B[a]P
100 by indigenous soil microorganisms. This is also the first study to investigate the effects
101 of concentration and repeated exposures of complex contaminant mixtures on the
102 development of B[a]P catabolic activity in soil.
103

104 **2. Materials and methods**

105 *2.1. Materials*

106 Non-labelled benzo[a]pyrene (purity >99%) and [7-^{14}C]benzo[a]pyrene (13.8 mCi
107 mmol^{-1}, radiochemical purity >96%) were purchased from Amersham Corp., USA.
108 Goldstar liquid scintillation cocktail, 7-ml and 20-ml glass scintillation vials were
109 obtained from Meridian, UK. Carbosorb-E® and Permafluor-E® sample oxidizer
110 cocktails were obtained from Perkin-Elmer Life Sciences, USA and Combustaid® from
111 Canberra Packard, UK. Diesel oil (specific gravity 0.85, C-content 87%; information
112 from supplier) was obtained from a local BP fuel station in Lancaster, UK.
113 Hydroxypropyl-β-cyclodextrin (purity >96%) was obtained from Acros Organics,
114 USA. Chemicals for the minimum basal salts (MBS) solution were supplied by BDH

115 Laboratory Supplies and Fisher Chemicals, UK while nutrient agar and agar powder
116 were from Oxoid, UK. All other chemicals used are analytical grade.
117
118 *2.2. Soils amendment*
119 A Dystric Cambisol with no known history of contamination with petroleum
120 hydrocarbons was collected (Ah horizon; 5–20 cm) from Myerscough Agricultural
121 College (Lancashire, UK). Physicochemical and microbial properties of the soil are
122 presented in Table 1. The soil was air-dried to approximately 40% of water holding
123 capacity (WHC), sieved (<2 mm) and stored at 4 °C until required. Prior to start of
124 acclimation experiment, the soil was equilibrated at 21 °C for 7 d and, thereafter,
125 moisture content was adjusted to 60% WHC. Aliquots of soil (500 g) were amended
126 with diesel to different amounts, as single or multiple applications, to achieve final
127 nominal concentrations of 500 or 5000 $mg_{oil\text{-}C}\ kg^{-1}{}_{soil}$ over a 20-week period, as
128 detailed in Table 2. The amendment of the soil with diesel followed the method
129 described by Doick et al., (2003). MBS solution (2 ml per 500 g of soil) was added to
130 all soils at every application time point in order to maintain high microbial viability;
131 the composition of the MBS solution has been described elsewhere (Fenlon et al.,
132 2011). After each amendment regime, the soils were returned into pre-cleaned amber
133 glass jars, sealed with perforated aluminium foil and allowed to acclimatize in darkness
134 at 21 ± 1 °C, until the next amendment time. At the end of 150 d acclimation, the
135 amended soils were sampled and analysed, as described in the following sections.
136
137 *2.3. Microbial respiratory activity and biomass-C determination*
138 Microbial respiratory activity within the diesel-amended soils was quantified by
139 monitoring CO_2 respiration rate in an aerobic closed static system in a MicroOxymax

140 respirometer (Multiple-sensor CO_2/O_2 10 Chamber System, Columbus). Triplicate
141 subsamples (20 g dry wt) were placed into 250-ml Schott bottles and fitted to the
142 MicroOxymax channels to measure background (basal) and substrate-induced
143 respiration (SIR) respiration. The basal samples were treated similarly to the SIR
144 samples expect that glucose (1.08 mg-C g^{-1}_{soil} dry wt) was not added. The initial
145 maximum SIR rate (2–4 h) was used to estimate the "active" glucose-responsive
146 biomass-C (Anderson and Domsch 1978). The metabolic quotient (qCO_2) was
147 calculated as the ratio of basal respiration to biomass-C (Wardle and Ghani 1995).
148
149 *2.4. Enumeration of B[a]P-degrading bacteria numbers*
150 After 150-d acclimation, B[a]P-degrading bacteria in soils were quantified by
151 measuring colony forming units (CFUs) following standard microbiological procedures
152 from serial dilutions of 10^{-1} to 10^{-3} (Lorch et al., 1995). Soil (2 ± 0.2 g) was extracted
153 with quarter strength Ringer's solution in a 1:10; 1 ml of the aliquot was serially
154 diluted with Ringer's solution. The resultant solution (0.1 ml) was spread on agar
155 plates impregnated with B[a]P (5 mg l^{-1}) as sole source of carbon and incubated at 25 ±
156 1 °C; distinct colonies were counted after 12–14 d.
157
158 *2.5. Soil amendment and sequential extractions of ^{14}C-benzo[a]pyrene in sterilised*
159 *amended soils*
160 Following acclimation, the amended soils were spiked with $^{12}C/^{14}C$-B[a]P standards
161 prepared in toluene to deliver a B[a]P concentration of 10 mg kg^{-1} with an associated
162 ^{14}C-activity of ~83 kBq kg^{-1}. To minimise the effect of toluene, the B[a]P standard was
163 added to ¼ of the soil and allowed to vent before adding the remaining soil (Doicket
164 al., 2003). The amended soils were sterilised by a series of autoclaving–re-incubation

three times over three alternate days. The effectiveness of this procedure was previously determined by plating out 1 g of soil on nutrient agar and potato-dextrose gar and incubating at 25 °C for 7–10 d; the method was chosen because it has the least impact on soil particle stability as compared to others (Kelsey et al., 2010). The spiked soil subsamples (1.25 g; $n = 3$) were place in Oak Ridge Teflon® centrifuge tubes and 5 ml of sterile deionised water added; tubes were then incubated upright at 21 °C for 5 d with gentle agitation (40 rpm; SANYO Gallenkamp orbital incubator). This allowed extensive equilibration and partitioning of B[a]P molecules between aqueous and solid phases. An aliquot (1 ml) of sodium azide (10 g l^{-1}) was added to maintain sterility during this period.

Aqueous extraction was carried out by adding 25 ml 0.01 M $CaCl_2$ solution to each centrifuge tube and incubated in an end-over-end position on a flatbed shaker (150 rpm) for 22–24 h. Following centrifugation (3000 x *g* for 1 h), 5 ml was withdrawn and mixed with 15 ml liquid scintillation cocktails in a 20-ml vial. The rest of the supernatant was carefully decanted and quantified, soil drained and 25 ml solution of 50 mM HP-β-CD was added to the tube. The processes of shake-extraction for 22–24 h and centrifugation and sampling of supernatant were repeated. The spent supernatant was discarded and replaced with 25 ml of distilled water for 10 min and centrifuged; this was to ensure the complete removal of remaining HP-β-CD solution. The ^{14}C-activity in both supernatants was summed up. Alkaline extraction of the HP-β-CD-extracted pellets to quantify fulvic acid/humic acids fractions (FA/HA-associated) was carried out until clear pale-yellow supernatant was obtained. The first extraction was with 30 ml $Na_4P_2O_7$:NaOH (1:20) and subsequent extractions with 20 ml $Na_4P_2O_7$:NaOH (1:1) (Macleod and Semple 2003). The ^{14}C-activity from all extracted samples was quantified by liquid scintillation counting (LSC) (Camberra Packard Tri

190 Carb 2300TR, Camberra Packard, UK) using standard calibration and quench
191 correction techniques and appropriate protocols.
192 The extracted soil pellets were allowed to dry under fume hood, weighed (*ca.* 1 g) into
193 cellulose combustion cones and combusted (3 min) with the aid of Combustaid® (200
194 µl) (Packard 307 Sample Oxidiser). The $^{14}CO_2$ was trapped with Carbosorb-E® (10 ml)
195 and Permafluor-E® (10 ml) used as a scintillation cocktail. The trapping efficiency
196 determined prior to sample combustion was >96%. Quantification of ^{14}C-activity in the
197 extracted soil pellets to estimate the fractions strongly-bound (solvent-nonextractable)
198 to humin materials (HM-associated) followed sample oxidation and LSC quantification
199 as previously described.
200
201 *2.6. Mineralisation of ^{14}C-benzo[a]pyrene in amended soils*
202 The aim here was to evaluate the effect of increasing HP-β-CD concentrations on ^{14}C-
203 B[a]P mineralisation in diesel-amended soils. Further, the effect of repeated exposures
204 to diesel on the development of microbial B[a]P catabolism was also assessed. The
205 standard radiorespirometric assay described by Reid et al., (2001), was used to monitor
206 ^{14}C-B[a]P mineralisation for a period of 30 d. The slurry-shake respirometry assay
207 consisted of 10 ± 0.2 g soil, sterile MBS solution (30 ml) and HP-β-CD (0, 12.5, 25
208 and 50 mM). The B[a]P standard was delivered in 5 µl toluene (per respirometer) as 10
209 mg ^{12}C-B[a]P kg^{-1} with an associated ^{14}C-activity of ~83 kBq kg^{-1}. The respirometers
210 were incubated on an orbital shaker (Janke and Kunkel, IKA®-Labortechnik KS250,
211 Germany) at 100 rpm under controlled laboratory conditions (temperature 21 ± 1 °C;
212 relative humidity 45%). During microbial catabolism of ^{14}C-B[a]P, the $^{14}CO_2$ trapped
213 with 1 M NaOH (1 ml) in a 7-ml vial was mixed with 5 ml scintillation cocktail and
214 quantified by LSC.

215

216 *2.7. Data presentation and statistical analysis*

217 The cumulative extents (%), maximum rates (% d^{-1}) and lag phases (d, time before
218 extent of $^{14}CO_2$ exceeds 5%) of mineralisation were calculated from the mineralisation
219 data. The graphs of the mineralisation data were prepared with SigmaPlot 10 for
220 Windows (SPSS Inc., Chicago, IL, USA). Analyses of variance (ANOVA) followed by
221 Holm-Sidak test, where there was statistical significance ($P < 0.05$) was performed
222 using SigmaStat ver. 3.5 (SPSS Inc.). The two main factors considered were diesel
223 concentration and HP-β-CD concentration.

224

225 **3. Results and discussion**

226 *3.1. Soil physicochemical and microbiological indices*

227 Compared to the unamended soil, the B[a]P-degrading bacterial CFUs significantly
228 increased in all of the amended soils with the values being higher in the 5x100 mg kg^{-1}
229 and 5x1000 mg kg^{-1} soils, after acclimation for 150 d (Table 3). For example, the
230 bacterial CFUs increased from <0.1 x 10^4 in the control soil to 12.5 x 10^4 in the 1x500
231 mg kg^{-1} soil to 28.6 x 10^4 in the 2x500 mg kg^{-1} soil and to 65.2 x 10^4 in the 5x100 mg
232 kg^{-1} soil, respectively. This is indicative of the sustained ability of soil microflora to
233 utilise B[a]P due to repeated exposures to diesel. Initial rapid increases in the relative
234 abundance of hydrocarbon-utilising bacteria have been observed in response to diesel
235 addition to soil (Peña et al., 2007). However, to maintain the ability of the microflora to
236 utilise non-growth substrates like B[a]P requires that adequate amounts of co-substrates
237 are frequently supplied (Cheung and Kinkle 2005; Fournier et al., 1981; Robertson and
238 Alexander 1994). For example, Robertson and Alexander (1994) demonstrated that the
239 higher populations of 2,4-D- or glyphosate-degrading microorganisms were sustained

240 for longer periods in soils amended twice at 10-d intervals than in soils amended once
241 with 10 mg 2,4-D or 20 mg glyphosate kg^{-1}.
242 The observation that the sizes of the microbial biomass-C (ranged from 1002 to 1708
243 $\mu g\ g^{-1}$) were not significantly different ($P > 0.05$) from the unamended soil (1521 $\mu g\ g^{-1}$)
244 after 150 d acclimation (Table 3) is consistent with Joergensen et al., (1995) who
245 also found that microbial biomass-C content of a control soil and that of the 0.5%-oil-C
246 treatment showed some fluctuations, but no marked changes during a 165-d incubation.
247 The basal respiration rate was 2.03 $\mu g\ g^{-1}\ h^{-1}$ in the unamended soil and significantly
248 increased ($P < 0.05$) to 2.30, 3.05 and 4.00 $\mu g\ g^{-1}\ h^{-1}$ in the 1x500, 2x250 and 5x100
249 mg kg^{-1} soils, respectively; similarly, SIR rates were significantly higher ($P < 0.05$) in
250 the amended soils than in the unamended soil. Overall, the results are indicative of an
251 increased microbial activity (i.e. higher qCO_2) in response to repeated exposures to
252 source of utilisable carbon substrates (Margesin et al., 2003; Towell et al., 2011b). For
253 example, Margesinet al., (2003) reported increased respiratory activity when a
254 petroleum hydrocarbon contaminated portion of an Alpine soil is compared with the
255 uncontaminated area. In cable oil spiked soils, microbial respiratory activity
256 significantly increased with increasing oil–soil concentrations, along with the number
257 of oil degraders (Towellet al., 2011b). Increased CO_2 production in a contaminated soil
258 has been attributed to soil microorganisms being able to utilise diesel as substrate for
259 growth and energy requirements (Joergensenet al., 1995).
260
261 *3.2. HP-β-CD-enhanced solubilisation of ^{14}C-benzo[a]pyrene in soil*
262 Overall, the soil treatments (i.e. frequency and rate of diesel application) did not
263 significantly affect ($P > 0.05$) B[a]P aqueous solubility, HP-β-CD-enhanced
264 solubilisation or its partitioning into different soil organic components (Table 4). The

265 amounts of ^{14}C-B[a]P in the aqueous phase were generally negligible (0.49–1.38%
266 corresponding to 1.6–4.6 μg l^{-1}), though slightly higher in the amended compared to
267 the unamended soil. B[a]P is known to have very low water solubility (3.8 μg l^{-1})
268 (Juhasz and Naidu 2000). The amounts extractable by HP-β-CD solution, usually
269 referred to as the bioaccessible fraction (Reid et al., 2000), were *ca.* ⅓ of the added ^{14}C-
270 B[a]P (27.36–35.01%, corresponding to 90–116 μg l^{-1}). A number of studies have
271 reported HP-β-CD-enhanced B[a]P solubilisation from liquid and solid matrices (Male
272 et al., 1995; Rafinet al., 2009; Towell et al., 2011a; Veignie et al., 2009). The results
273 are comparable with those of Towellet al., (2011a), who reported that 21.5–24.3% of
274 50 mg kg^{-1} B[a]P was solubilised in the presence of 50 mM HP-β-CD for the same soil
275 investigated. There was no marked difference between the amount of ^{14}C-B[a]P that
276 partitioned to the humic/fulvic acids (30.96–37.04%) and to the humin (28.90–39.80%)
277 components of soil; diesel amendment had no effect on the partitioning too.
278 In this study, the increase in apparent aqueous dissolution of B[a]P translates to
279 solubility enhancement factors (SEF: ratio of B[a]P fraction in HP-β-CD solution to
280 that in aqueous phase) ranging from 26 to 67. Values of SEF in the literature ranged
281 widely, depending on the type and concentration of the target (guest) contaminant and
282 type of CD, as well as whether the dissolution was from a liquid or solid matrix. For
283 example, at the same CD concentration, the SEF value for a PAH is always higher in
284 the presence of HP-β-CD than in either βCD or γCD (Gao et al., 2013). While βCD
285 produces higher SEF values for ≤3-ring PAHs, it gives lower values for ≥4-ring PAHs
286 compared with γCD (Badr et al., 2004; Berselliet al., 2004; Carroll and Brusseau 2009;
287 Maleet al., 1995). In general, for a particular cyclodextrin, SEF values appeared to
288 increase as ring number of PAH increases (Luong et al., 1995). Compared with their
289 aqueous solubility, the solubility of PAHs in HP-β-CD is enhanced 224-fold and 7500-

290 fold for naphthalene and B[a]P, respectively, with other PAHs yielding values between
291 these limits (Luonget al., 1995).
292
293 *3.3. Effects of single or repeated exposures on ^{14}C-benzo[a]pyrene mineralisation in*
294 *soil*
295 The indices (lag phase, maximum rate and extent) of ^{14}C-B[a]P mineralisation indicate
296 that the extant soil microflora have inherently poor ability to mineralise B[a]P (Figure
297 1A; Table 5). This observation is consistent with previous reports on this soil (Stroud et
298 al., 2007; Towellet al., 2011a) and other unexposed soils (Carmichael and Pfaender
299 1997; Grosser et al., 1991). The relatively low abundance of degrader populations and
300 inadequate assemblages of competent consortia in soil have been implicated in poor
301 B[a]P mineralisation (Kanaly and Harayama 2000). In addition, the inability of B[a]P
302 to readily support microbial growth, the requirement for certain co-substrates for
303 effective degradation and the unfavourable physicochemical properties can limit B[a]P
304 mineralisation in soil (Bamforth and Singleton 2005).
305 Overall, regardless of frequency and rate of application, acclimation to diesel
306 significantly ($P < 0.05$) enhanced B[a]P catabolic activity of soil, as indicated by the
307 relative shorter lag phases and higher maximum rates and extents of mineralisation
308 (Figure 1B–G; Table 5). In general, the lag phase reduced from >30 d to 10.33–22.48
309 d, the maximum rate increased from 0.06% d^{-1} to 0.54–3.25% d^{-1} and the extent of ^{14}C-
310 B[a]P mineralisation increased from 0.74% to 7.92–40.48% in the unamended soil and
311 for all the amended soils, respectively. The results are in agreement with observations
312 made by other investigators (Grosseret al., 1991; Kanalyet al., 1997). Mineralisation
313 has been reported to proceed after lag periods of several weeks to maximum extents of
314 4 to 25% of ^{14}C-B[a]P in various contaminated soils after 180 d (Grosseret al., 1991).

315 Significant mineralisation of up to 50% of added ^{14}C-B[a]P was recorded after 150 d in
316 a soil amended with crude oil (Kanalyet al., 1997). In terms of other compounds,
317 significantly reduced lag phases and higher rates and extents of ^{14}C-pyrene
318 mineralisation resulted after 8 and 76 weeks of pre-exposure to 100 mg pyrene kg^{-1} in a
319 pasture and woodland soil, respectively (Macleod and Semple 2002). Similarly,
320 transformer oil (0.01–0.1% w/w) enhanced the ability of indigenous soil microflora to
321 mineralise ^{14}C-phenanthrene after acclimation for 28 d (Lee et al., 2003). Spain et al.,
322 (1980) observed faster rate of ^{14}C-labeled methyl parathion or *p*-nitrophenol
323 mineralisation in pre-exposed cores than in control cores and attributed this to
324 adaptation of microorganisms to the contaminants. However, it were not in all cases
325 that acclimation resulted in higher extents of mineralisation. For example, Swindell and
326 Reid (2006) reported that diesel (0–2000 mg kg^{-1}) did not enhance phenanthrene
327 mineralisation further even after soil was acclimated for 225 d.
328 In comparison, repeated exposure to diesel at low concentration (2x250 mg kg^{-1} or
329 5x100 mg kg^{-1}) enhanced ^{14}C-B[a]P mineralisation more than single exposure at low
330 concentration (1x500 mg kg^{-1}), whereas single exposure to high concentrations
331 (1x5000 mg kg^{-1}) enhanced mineralisation more than repeated exposure to high
332 concentrations (2x2500 mg kg^{-1} or 5x1000 mg kg^{-1}) (Table 5). For instance, the extents
333 of mineralisation were 40.48 and 17.72% in the 2x250 mg kg^{-1} and 1x500 mg kg^{-1}
334 soils, respectively; while they were 21.95 and 7.92% in the 1x5000 mg kg^{-1} and
335 2x2500 mg kg^{-1} soils, respectively. This finding is remarkable because it indicates that
336 the interplay of the exposure concentration and interval between exposure events is a
337 determinant factor for the development of B[a]P catabolic activity in soil. No previous
338 report in terms of the effect of frequency of exposure on the development of B[a]P
339 catabolic activity is available in the literature. However, aspects of the results from this

340 study are comparable with the findings of studies on other PAHs and pesticides. For
341 example, Macleod and Semple (2006) observed that multiple amendments (2x50 or
342 4x50 mg kg^{-1} treatments) led to apparently shorter lag phases of ^{14}C-pyrene
343 mineralisation relative to single amendment of soil with pyrene (1x100 or 1x200 mg
344 kg^{-1} treatments). Pre-exposure of soil microorganisms for 1 or 6 weeks to a low level of
345 pyrene in the presence of phenanthrene enhanced pyrene degradative potentials to the
346 levels of that in soil pre-exposed to a higher level of pyrene (Cheung and Kinkle 2005).
347 By comparing soils from different agricultural sites which had an history of repeated
348 atrazine applications or no recorded atrazine application, at least for the last three
349 years, Yassir et al., (1999) reported that accelerated mineralisation of atrazine was as a
350 result of prior exposure to multiple application of the herbicide. The authors found that
351 the unexposed soils showed low degradative potentials for atrazine. Previous, it has
352 been suggested that in sediment-water cores in which exposure is chronic, the rate of
353 biodegradation might be very rapid compared with that in areas receiving intermittent
354 exposure (Spainet al., 1980). In contrast, the results of this study suggest that
355 adaptation through repeated exposures will support faster rate and higher extent of
356 mineralisation than single chronic exposure, especially where the contaminant is not an
357 obligate growth substrate and/or requires co-substrates for it degradation. In tandem
358 with our submission, Fournieret al., (1981) had suggested that there is a possibility of
359 the loss of catabolic activity after a period of time if such substrates that sustain growth
360 of the cometabolic degraders of 2,4-D are no longer available in the environment.
361

362 *3.4. Presence and effect of HP-β-CD concentration on ^{14}C-benzo[a]pyrene*
363 *mineralisation in soil*

364 Several studies have demonstrated significantly HP-β-CD-enhanced biodegradation of
365 aliphatic hydrocarbons and PAHs with up to four fused benzene rings (e.g. Allan et al.,
366 2007; Bardi et al., 2000; Garon et al., 2004; Molnar et al., 2005; Reid et al., 2004;
367 Steffan et al., 2002; Wang et al., 2005; Wang et al., 1998), but information regarding
368 biodegradation of PAHs with five or more rings is limited in the literature (Allan et al.,
369 2007; Cuypers et al., 2002; Ye et al., 1996). For example, Wang et al., (1998) showed
370 that HP-β-CD addition significantly enhanced solubilisation and the rate of
371 phenanthrene degradation by *Burkholderia* sp. CRE 7. Only 0.3% of the added
372 phenanthrene remained at the end of a 48-h incubation in the presence of 100 g l^{-1} HP-
373 β-CD compared to 45.2% in the absence of HP-β-CD. It has been reported that 100 g l^{-1}
374 HP-β-CD improved the efficiency of degradation of some PAHs and phenolic
375 compounds from three municipal gas plant site soils by catabolically active PAH and
376 phenol-degrading microorganisms or indigenous soil microbiota (Allan et al., 2007).
377 Degradation of pyrene by *Burkholderia* sp. CRE 7 was initiated after approximately 15
378 weeks in the presence of 100 g l^{-1} HP-β-CD and reached a maximum extent of 14%
379 after 22 weeks (Wang et al., 2005).

380 Meanwhile, HP-β-CD failed to further increase mineralisation of B[a]P by
381 *Sphingomonas paucimobilis* (Ye et al., 1996) or by an active microbial consortium
382 (Cuypers et al., 2002). This is agreement with the results in the present study; in
383 general, the addition of HP-β-CD did not have a consistent effect on any aspect of ^{14}C-
384 B[a]P mineralisation (Table 5). For example, in the microcosms with 50 mM HP-β-
385 CD, the extents of mineralisation were significantly higher ($P < 0.05$) for the control
386 and 2x2500 mg kg^{-1} soils; significantly lower ($P < 0.05$) for the 1x500 mg kg^{-1}, 1x5000

387 mg kg^{-1} and 2x200 mg kg^{-1} soils; and similar ($P > 0.05$) for the 5x100 mg kg^{-1} and
388 5x1000 mg kg^{-1} soils, as compared to incubations without HP-β-CD. This
389 inconsistency in the effect was also observed for assays with 12.5 and 25 mM HP-β-
390 CD. As earlier mentioned, HP-β-CD significantly increased B[a]P apparent solubility
391 (26 to 67 times) and the acclimation to diesel significantly enhanced (11 to 55 times)
392 B[a]P catabolic activity in soil. Hence, it was expected that HP-β-CD addition to the
393 amended soils would improve the overall efficiency of B[a]P mineralisation; however
394 it was not so.
395 Although a large apparent solubility means more B[a]P molecules are made *accessible*
396 to microorganisms in solution by HP-β-CD but the results of the mineralisation
397 experiment indicate the complexed B[a]P molecules are not *available* to
398 microorganisms. Semple et al., (2004) provide an excellent critique of the concepts of
399 bioaccessibility and bioavailability and the implications to biodegradation of
400 hydrophobic organic contaminants. The amount of a chemical that is bioavailable is
401 believed to depend on (i) the rate of mass transfer from the soil to the living cell and
402 (ii) the rate of uptake and metabolism via a specific biochemical pathway (Semple et
403 al., 2003). On this basis, the finding of this present study is interpreted as the result of
404 HP-β-CD-imposed limitations to cellular uptake and biodegradation of the complexed
405 B[a]P by active microbial cells. Based on the current data from this study, there is
406 currently no clear explanation as to why there was negligible biodegradation of
407 complexed B[a]P. However, it is hypothesized that the limitation to *microbial*
408 *availability* of B[a]P is due to the nature of inclusion complexes formed by HP-β-CD.
409 So far the limited evidence of HP-β-CD-enhanced B[a]P biodegradation has been in
410 combination with other chemical treatments (Rafin et al., 2009; Veignie et al., 2009).
411 For example, Rafin et al., (2009) reported a B[a]P biodegradation of 8% in presence of

412 HP-β-CD compared to 5% in the absence of HP-β-CD by *Fusarium solani* after 12 d
413 incubation. When combined with Fenton's reagent, 25% degradation by *Fusarium*
414 *solani* was recorded in the presence of HP-β-CD.
415 Further, despite increases in B[a]P solubility with increasing HP-β-CD concentration
416 (Berselli et al., 2004; Male et al., 1995), the results of this study indicate that the
417 enhancing effect of increasing HP-β-CD concentration on ^{14}C-B[a]P mineralisation
418 was minimal and inconsistent (Table 5). At the highest concentration (50 mM)
419 investigated, extents of ^{14}C-B[a]P mineralisation were generally lower; this being
420 significant in some cases (e.g., 1x500 mg kg^{-1}, 1x5000 mg kg^{-1} and 2x250 mg kg^{-1}
421 soils), as compared with systems without HP-β-CD. Interestingly, however, slight but
422 significantly higher extents of mineralisation were observed at the lowest HP-β-CD
423 concentration (12.5 mM), especially in amended soils with enhanced catabolic activity.
424 In the 1x500 mg kg^{-1}, 1x5000 mg kg^{-1} and 2x250 mg kg^{-1} soils the extents of
425 mineralisation were 30.36, 25.35 and 50.20%, respectively, in the presence of 12.5 mM
426 HP-β-CD and decreased to 29.36, 14.11 and 36.13%, respectively, in the presence of
427 25 mM HP-β-CD and further decreased to 13.13, 14.13 and 27.44%, respectively, in
428 the presence of 50 mM HP-β-CD. Few studies have examined the effect of HP-β-CD
429 concentration on biodegradation of contaminants (Male et al., 1995; Steffan et al.,
430 2002; Stroud et al., 2009; Wang et al., 1998). The results of previous studies agree with
431 our findings; for example, it was demonstrated that lignin peroxidase (LiP) readily
432 oxidized B[a]P when complexed with HP-β-CD concentration of up to 100 mM but
433 50% inhibition occurred at a concentration of 250 mM HP-β-CD (Male et al., 1995). A
434 50% enzyme inhibition was observed for the oxidation of pyrene at 40 mM HP-β-CD,
435 and complete inhibition at 100 mM HP-β-CD (Male et al., 1995). The higher

436 concentration of HP-β-CD promoted a faster degradation of phenanthrene within the
437 first 2 d, although overall extents of mineralisation were similar after 7 d in the
438 presence of 10 and 100 mg l^{-1} HP-β-CD (Wang et al., 1998). Although not statistically
439 significant ($P > 0.05$), the extents of ^{14}C-phenanthrene mineralisation were higher in
440 the presence of 20 mM than in 10 mM HP-β-CD, but lower in the presence of 40 mM
441 HP-β-CD than for the other two concentrations (Stroud et al., 2009). Moreover, HP-β-
442 CD concentrations (10–40 mM) have no effect on ^{14}C-hexadecane mineralisation
443 (Stroud et al., 2009). Similar to the observation in this study, Steffan et al., (2002) have
444 showed that among other variables, the composition of indigenous microbial
445 populations influenced the rate of mineralisation of HP-β-CD solubilised dodecane.
446 Ko et al., (1999) demonstrated that the formation of HP-β-CD–guest inclusion complex
447 is a very rapid process, with over 95% of the partitioning to HP-β-CD occurring within
448 10 min. This spontaneous mass transfer of somewhat high B[a]P concentration into the
449 aqueous phase and/or increased retention of polar but more harmful metabolites in
450 solution may increase toxicity to microbiota and cause inhibition or reduction of
451 expression of enzyme activity and even death of susceptible organisms. Eventually,
452 this could lead to a substantial change within the microbial community structure, thus
453 altering the biodegradation process and/or fate of the contaminant. This plausibly
454 explains the decreased ^{14}C-B[a]P mineralisation as HP-β-CD concentration increased.
455 The ecological and ecotoxicological implications are that enhanced catabolic potential
456 is more important than enhanced bioavailability to B[a]P biodegradation, and increased
457 B[a]P solubilisation by HP-β-CD without further mineralisation poses a serious risk to
458 soil biota and underground aquifers.
459

4. Conclusions

Overall, the data presented here indicate that the presence of catabolically-competent microorganisms has a greater effect than enhanced bioavailability to expedite B[a]P mineralisation in soil. Also, the data provide some understanding of the variability in the effects of chronic exposure to diesel on the development of B[a]P catabolic activity as well as the implications of enhancing B[a]P aqueous solubility with HP-β-CD during bioremediation of oil-contaminated soils. To the authors' knowledge, this is the first study of the effect cyclodextrin on B[a]P degradation by indigenous soil microorganisms. These findings are important for assessing the fate of B[a]P and designing bespoke remediation strategies for HMW-PAHs in soils that are susceptible to periodic contamination with petroleum oils. Further research is needed to understand the influence of specific soil biotic and abiotic properties. Studies should also investigate the effects of different cyclodextrins on B[a]P, especially in the presence of either readily-degradable PAHs or highly-recalcitrant PAHs as co-contaminants. Because this study monitored the degradation of freshly added B[a]P in "artificially" contaminated soil, future studies will use field-contaminated soils with variable histories of chronic exposures to petroleum oils.

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651

652 **Tables**

Table 1: Physicochemical and microbial characteristics of Myerscough soil.

Characteristics		Values	Characteristics	Values
Soil classification		Dystric Cambisol	Total bacterial (CFU g^{-1})	3.63×10^5
Grid reference		SD496402	Benzo[a]pyrene (CFU g^{-1})	$<1 \times 10^3$
Texture		Sandy loam	PAHs (µg kg^{-1})	
Particle size analysis	Sand Course	17.1	Naphthalene	1.07
(g kg^{-1})	Medium	155.6	Acenaphthylene	ND
	Fine	383.6	Acenaphthene	ND
	Total	556.3	Fluorene	0.58
	Silt	249.6	Phenanthrene	17.70
	Clay	194.1	Anthracene	9.03
pH	dH_2O	6.53	Fluoranthene	ND
	$CaCl_2$	5.18	Pyrene	ND
Elemental analysis	Extractable P	26	Benzo[a]anthracene	ND
(mg kg^{-1})	Available K	143	Chrysene	0.41
	Available Mg	579	Benzo[*b*]fluoranthene	0.13
	Ca	598	Benzo[*k*]fluoranthene	0.50
	Na	57	Benzo[*a*]pyrene	ND
	Cu	3	Indeno[1,2,3-*cd*]pyrene	0.12
	Fe	269	Dibenz[*ah*]anthracene	ND
	Pb	4	Benzo[*ghi*]perylene	ND
Soil organic matter (%; LOI)		4.82	∑16 US EPA PAHs	29.54
Total organic carbon (mg g^{-1})		17.0		
Total nitrogen (mg g^{-1})		1.4		

653 LOI: Loss on ignition, 450 °C for 24 h

Table 2: Soil amendment regimes, acclimation durations and soil slurry microcosm set-ups.

Treatments ($mg_{oil\text{-}C}$ kg^{-1}_{soil})	Timing of diesel applications (days) prior to start of experiment					
Rate[a] x Concentration[b]	-150	-115	-80	-45	-10	0
0 (control)						Fresh $^{12/14}$C-B[a]P (10 mg kg^{-1}; *ca.* 83 Bq g^{-1} soil) ± HP-β-CD (0, 12.5, 25 and 50 mM) added to slurry microcosms
1x500	√					
1x5000	√					
2x250	√		√			
2x2500	√		√			
5x100	√	√	√	√	√	
5x1000	√	√	√	√	√	

[a] Number (rate) of diesel applications
[b] Added concentration at each application

Table 3: Some microbiological indices of the diesel-amended soils.[§]

Treatments ($mg_{oil\text{-}C}\ kg^{-1}_{soil}$) Rate[a] x Concentration[b]	B[a]P Degraders (x 10^4 CFU g^{-1}_{soil})	Respiratory Responses (μg g^{-1} h^{-1}) Basal[†]	SIR[ǂ]	Biomass-C (μg g^{-1}_{soil})[#]	Metabolic Quotient, qCO_2 (μg C-CO_2 mg^{-1} biomass-C h^{-1})
0 (control)	< 0.1	2.03 ± 0.10[a‡]	195.87 ± 1.28[a]	1521.11 ± 1.88[dc]	1.94 ± 0.02[a]
1x500	12.5 (3.75)	2.30 ± 0.30[ab]	246.73 ± 2.57[bc]	1437.68 ± 39.00[c]	2.22 ± 0.04[a]
1x5000	23.6 (1.2)	2.00 ± 0.10[a]	293.90 ± 1.43[d]	1001.82 ± 38.89[a]	4.87 ± 0.18[d]
2x250	28.6 (6.33)	3.05 ± 0.20[b]	260.94 ± 5.93[c]	1098.06 ± 6.22[ab]	2.95 ± 0.05[b]
2x2500	15.0 (3.63)	3.02 ± 0.05[b]	238.95 ± 12.65[bc]	1253.99 ± 21.15[bc]	3.70 ± 0.08[c]
5x100	65.2 (5.8)	4.00 ± 0.20[c]	220.93 ± 7.82[b]	1707.93 ± 48.33[d]	1.85 ± 0.06[a]
5x1000	87.6 (3.58)	4.70 ± 0.35[c]	212.12 ± 1.49[b]	1400.51 ± 82.55[c]	2.87 ± 0.17[b]

§ Amended soils were analysed after 150 d of ageing.
† Values (mean ± SEM) are average of 2-hourly continuously measurements for 24 h in triplicate samples.
ǂ SIR is the maximum glucose-induced respiration within 24 h.
"Active" glucose-responsive biomass-C.
‡ Different lower-case letters down the column are statistically significant (P <0.05).
[a] Number (rate) of diesel applications
[b] Added concentration at each application

668

669 Table 4: ^{14}C-B[a]P partitioning in the diesel-amended soil.[§]

Treatments ($mg_{oil\text{-}C}$ kg^{-1}_{soil}) Rate[a] x Concentration[b]	Aqueous (%)	HP-β-CD (%)	FA + HA (%)	Humin (%)	Total recovery (%)	SEF[#]
0 (control)	0.49 ± 0.25[†]	32.75 ± 2.28	36.03 ± 2.63	31.70 ± 1.61	100.96 ± 5.00	67
1x500	0.74 ± 0.15	28.91 ± 4.15	32.39 ± 5.09	33.09 ± 8.94	95.13 ± 10.31	39
1x5000	1.30 ± 0.36	33.90 ± 2.28	34.15 ± 2.19	32.24 ± 1.44	101.58 ± 4.56	26
2x250	0.68 ± 0.15	35.01 ± 0.41	30.96 ± 2.86	28.90 ± 5.03	95.55 ± 4.80	51
2x2500	1.58 ± 0.25	27.36 ± 3.89	37.04 ± 6.69	33.02 ± 5.20	98.80 ± 11.25	20
5x100	0.54 ± 0.07	30.48 ± 1.29	34.72 ± 1.29	39.80 ± 2.81	105.43 ± 4.36	56
5x1000	0.70 ± 0.18	32.95 ± 3.02	33.48 ± 5.91	39.51 ± 6.74	106.65 ± 9.97	47

670 § Amended soils were analysed after 150-d of ageing. Diesel amendment did not significantly influenced (P >0.05) B[a]P partitioning.
671 † Values are means ± SEM of three independent replicates.
672 # Solubility enhancement factor
673 [a] Number (rate) of diesel applications
674 [b] Added concentration at each application
675 FA + HA: Fulvic/humic acids
676

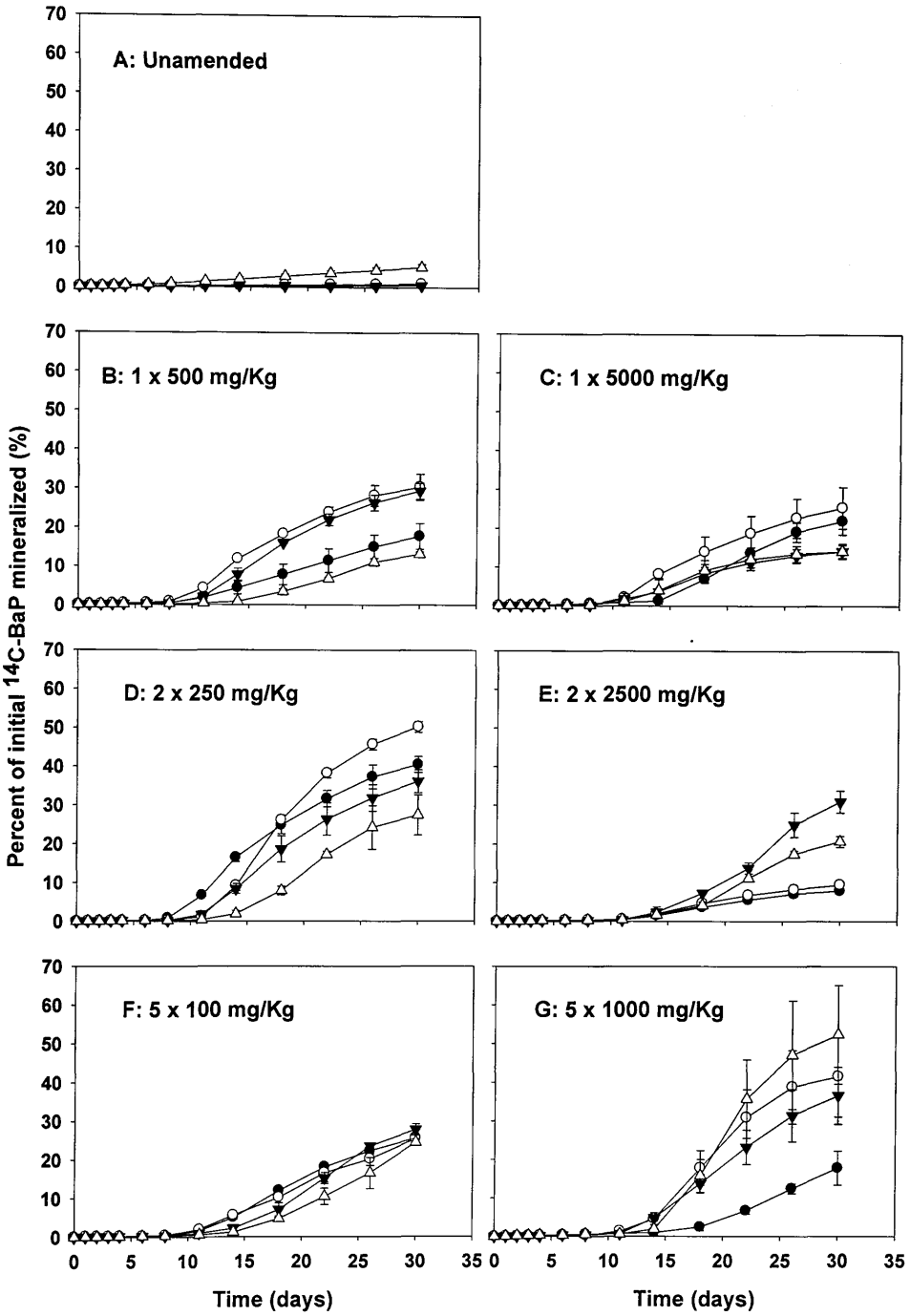
677

678 Table 5: Effects of HP-β-CD concentrations on mineralisation of ^{14}C-B[a]P (10 mg kg^{-1}) in diesel-amended soils.

Parameters	HP-β-CD	Soil treatment (number of application x rate) ($mg_{oil\text{-}C}$ kg^{-1}_{soil})						
	(mM)	0 (control)	1x500	1x5000	2x250	2x2500	5x100	5x1000
Lag phase	0	>30^{a‡}	15.71 ± 2.35^{c}	17.91 ± 1.96^{c}	10.33 ± 0.46^{b}	14.64 ± 0.10^{bc}	13.90 ± 0.33^{b}	22.48 ± 1.96^{d}
(d)	12.5	>30^{a}	14.36 ± 3.06^{b}	12.48 ± 0.30^{b}	12.97 ± 1.07^{b}	18.72 ± 1.41^{b}	13.53 ± 0.39^{b}	14.98 ± 1.69^{b}
	25	>30^{a}	14.16 ± 1.62^{b}	15.26 ± 2.05^{b}	13.35 ± 1.15^{b}	16.02 ± 0.39^{b}	16.54 ± 1.18^{b}	14.66 ± 1.16^{b}
	50	>24.47$^{\#a}$	20.98 ± 2.19^{b}	14.91 ± 1.94^{b}	16.97 ± 1.30^{b}	18.49 ± 1.86^{b}	20.05 ± 2.83^{b}	16.43 ± 1.97^{b}
Fastest rate	0	0.06 ± 0.01^{a}	0.91 ± 0.10^{b}	1.70 ± 0.20^{c}	3.25 ± 0.46^{d}	0.54 ± 0.53^{b}	1.73 ± 0.03^{c}	1.44 ± 0.89^{c}
(% d^{-1})	12.5	0.04 ± 0.01^{a}	2.54 ± 0.11^{c}	2.01 ± 0.24^{c}	4.25 ± 0.56^{e}	0.68 ± 0.13^{b}	1.59 ± 0.23^{c}	3.30 ± 2.00^{d}
	25	0.04 ± 0.01^{a}	1.69 ± 0.46^{c}	1.19 ± 0.18^{b}	2.57 ± 0.62^{c}	2.81 ± 1.73^{c}	2.07 ± 0.31^{bc}	2.37 ± 1.11^{b}
	50	0.16 ± 0.04^{a}	1.08 ± 0.25^{b}	1.30 ± 0.12^{b}	2.33 ± 0.72^{c}	1.77 ± 0.23^{b}	1.98 ± 0.88^{bc}	4.99 ± 2.36^{d}
Cumulative	0	0.74 ± 0.30^{a1†}	17.72 ± 3.34^{c2}	21.95 ± 3.52^{d2}	40.48 ± 2.06^{e2}	7.92 ± 0.33^{b1}	25.55 ± 0.37^{d1}	17.75 ± 4.44^{c1}
extent (%)	12.5	0.92 ± 0.24^{a1}	30.36 ± 3.36^{c3}	25.35 ± 5.40^{c2}	50.20 ± 1.37^{d3}	9.47 ± 0.93^{b1}	25.39 ± 0.95^{c1}	41.50 ± 10.41^{d1}
	25	0.49 ± 0.08^{a1}	29.36 ± 2.05^{c3}	14.11 ± 1.69^{b1}	36.13 ± 2.97^{c1}	31.00 ± 2.87^{c3}	27.74 ± 1.45^{c1}	36.55 ± 7.39^{c1}
	50	5.16 ± 0.85^{a2}	13.13 ± 0.55^{b1}	14.13 ± 1.95^{b1}	27.44 ± 5.19^{c1}	20.71 ± 1.40^{c2}	24.45 ± 0.81^{c1}	52.34 ± 12.77^{d1}

679 ‡ Different lower-case letters across the row are statistically different (P <0.05).
680 † Different superscript numbers down the column under the heading "cumulative extent" are statistically different (P <0.05).
681 # Only one of the triplicate samples reached >5% mineralisation before the end of the experiment.

682 **Figures**



683

684 Figure 1: Mineralisation of ^{14}C-B[a]P (10 mg kg^{-1}) in Myerscough soil (A) unamended
685 and amended with diesel oil at (B) 1x500 $mg_{oil\text{-}C}$ kg^{-1}_{soil}, (C) 1x5000 $mg_{oil\text{-}C}$ kg^{-1}_{soil}, (D)
686 2 x 250 $mg_{oil\text{-}C}$ kg^{-1}_{soil}, (E) 2 x 2500 $mg_{oil\text{-}C}$ kg^{-1}_{soil}, (F) 5 x 100 $mg_{oil\text{-}C}$ kg^{-1}_{soil} and (G) 5
687 x 1000 $mg_{oil\text{-}C}$ kg^{-1}_{soil}. Slurry microcosms were un-supplemented (•), or supplemented
688 with HP-β-CD at 12.5 mM (○), 25 mM (▼), and 50 mM (Δ). Error bars, where visible,
689 are SEM (n=3).

Paper VII

Influence of HP-β-CD-enhanced solubilisation and diesel-enhanced catabolic activity on benzo[a]pyrene biodegradation in four soils

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15 **Abstract**

16 The key challenges to benzo[a]pyrene biodegradation are low bioavailability and poor
17 catabolic potentials in soils. This paper evaluates the relative contributions of these
18 factors to benzo[a]pyrene biodegradation in four soils with differing biotic and abiotic
19 properties. The solubility-enhancement agent, HP-β-CD, significantly improved the
20 apparent aqueous dissolution of benzo[a]pyrene from soil matrices; effect decreased as
21 soil organic matter and clay contents increased. Overall, ^{14}C-benzo[a]pyrene
22 mineralisation was significantly enhanced in all soils pre-exposed to diesel (0.05 and
23 0.5% w/w for 150 d); this being greater at the higher diesel concentration. Addition of
24 fresh diesel (0.05%) to pre-exposed soils enhanced mineralisation further. However,
25 the presence of HP-β-CD reduced the extents of ^{14}C-benzo[a]pyrene mineralisation in
26 three of the soils. Results indicated that the presence of catabolically-competent
27 microorganisms and suitable co-substrates have greater effects than enhanced
28 bioavailability to facilitate extensive benzo[a]pyrene mineralisation. HP-β-CD-
29 enhanced solubilisation of benzo[a]pyrene without subsequent mineralisation may
30 increase the toxicity risk to underground aquifer.

31

32 **Capsule**:

33 HP-β-CD significantly increased the apparent solubility of B[a]P, but its presence
34 reduced the extents of B[a]P mineralisation in most soils.

35

36 **Keywords**: *Benzo[a]pyrene; Bioaccessibility; Biodegradation; Diesel oil; HMW*
37 *PAHs; Hydroxypropyl-β-cyclodextrin (HP-β-CD); Soil*

38

Highlights:

- ▶ Effects of bioaccessibility and degradative potentials on B[a]P catabolism in soils.
- ▶ HP-β-CD increased B[a]P aqueous dissolution; decrease as SOM or clay content increased.
- ▶ Pre-exposure to diesel enhanced B[a]P catabolism; increase as diesel level increased.
- ▶ Presence of fresh diesel further enhanced B[a]P catabolism in all amended soils.
- ▶ Presence of HP-β-CD reduced the extents of B[a]P mineralisation in most amended soils.

1. Introduction

Heterogeneous non-aqueous phase liquids (NAPLs), such as crude or refined petroleum oils, creosote and coal tar, consist of complex mixtures of hydrophobic organic compounds (HOCs), including aliphatic and polycyclic aromatic hydrocarbons (PAHs). Like other HOCs, the high molecular weight (HMW) PAH benzo[a]pyrene (B[a]P) has very low aqueous solubility (0.0038 mg l^{-1}) and strong affinity for hydrophobic and lipophilic matrices (log K_{ow} 6.06), which means that the PAH will readily sequester to soil organic matter (SOM) and clay minerals.[1, 2] Further, B[a]P is of particular ecotoxicological importance because it is highly toxic and exhibit carcinogenic and mutagenic properties; thus, it poses a great risk to soil biota and humans.[3] Therefore, aggressive treatments are needed to detoxify B[a]P in polluted soils and sediments.

The design and deployment of bespoke remediation technologies which take advantage of the catabolic activity of present within indigenous soil microbial communities at sites contaminated with NAPLs are on the increase. Many such technologies aim to improve dissolution of the contaminants from soil-solid phase by using solubility-enhancement agents (SEA), such as cyclodextrins, biosynthetic and chemically synthesised surfactants.[4-10] The solubilising effect of cyclodextrins may be less than most other SEAs; however, cyclodextrins have greater advantages for *in situ* bioremediation of contaminated soils because they are extremely water-soluble, do not form emulsions and are non-toxic and fairly biodegradable, thus posing no hazard to the ecosystem.[8, 11] Several studies have reported increased biodegradation of aliphatic hydrocarbons and PAHs with up to four fused benzene rings in the presence of HP-β-CD,[12-17] but information regarding biodegradation of PAHs with five or more rings is limited in the literature,[18] and so far no study has provided evidence for HP-β-CD-

74 enhanced mineralisation of B[a]P. Meanwhile, the apparent aqueous dissolution of
75 B[a]P has been shown to increase tremendously (up to 7,500 times) in the presence of
76 HP-β-CD in aqueous solution.[19]
77 Further, almost all of the previous laboratory studies have been carried out on isolated
78 microorganisms and mostly in liquid system with only a few in soil matrix. For
79 example, *Burkholderia* CRE 7 was used to investigate biodegradation of phenanthrene
80 and pyrene in aqueous solution[12, 13] while *Absidia cylindrospora* was used for fluorene
81 degradation in soil-slurry.[14] For greater environmental relevance, however, studies
82 should focus more on HMW-PAHs, such as B[a]P, because of their comparatively
83 lower aqueous solubility, and increased resistance to microbial attack, as well as higher
84 carcinogenic and mutagenic potentials. Moreover, particular interest should be directed
85 at using the indigenous microorganisms in soil because bioavailability is believed to be
86 organism- and indeed species-specific, with different PAH-degrading bacteria
87 inhabiting the same soil adapted to different PAH bioavailabilities.[20, 21] To the authors'
88 knowledge, studies of HP-β-CD-enhanced degradation of B[a]P by indigenous soil
89 microflora are rare in the literature.
90 Recently, the authors observed that increasing HP-β-CD concentrations had marginal
91 influence on B[a]P mineralisation by the indigenous microorganisms in a soil despite
92 significantly enhancing the apparent aqueous solubility of the compound (unpublished
93 data). Therefore, in this paper we compare the effects of HP-β-CD-enhanced
94 solubilisation and diesel-enhanced catabolic activity on B[a]P mineralisation in four
95 disparate soils. Because successful implementation of *in situ* bioremediation of PAHs
96 is contingent upon a good understanding of the effects of a variety of soil biotic and
97 abiotic factors on contaminant fate, there is need to know, in practical terms, how HP-
98 β-CD affects bioavailability and biodegradation in a wide range of soils.

2. Materials and methods

2.1. Materials

Non-labelled benzo[a]pyrene (>99%) and [7-^{14}C]benzo[a]pyrene (13.8 mCi mmol^{-1}, >96%) were purchased from Amersham Corp., USA. Goldstar liquid scintillation cocktail, 7-ml and 20-ml glass scintillation vials were obtained from Meridian, UK. Carbosorb-E® and Permafluor-E® sample oxidiser cocktails were obtained from Perkin-Elmer Life Sciences, USA and Combustaid® from Canberra Packard, UK. Diesel oil (specific gravity 0.85, C-content 87%) was acquired from a local BP petrol filling station in Lancaster, UK. Hydroxypropyl-β-cyclodextrin (>96%) was obtained from Acros Organics, USA. Chemicals for the minimum basal salts (MBS) solution were supplied by BDH Laboratory Supplies and Fisher Chemicals, UK. All other chemicals used are analytical grade.

2.2. Soils and soil amendment

One of the four soils used in this study was obtained from Livingstone Island, Antarctica while the other three were collected from various locations in Lancashire, UK (Table 1); the soils differed in their physicochemical characteristics. The soils were air-dried for 48 h to *ca.* 40% of their water holding capacity (WHC), sieved (≤2 mm) and stored at 4 °C until required. Aliquots of the soil (500 g) were amended with diesel following the one-step spiking/rehydration method described by Doick, et al.[22] to concentrations of 0, 500 and 5000 $mg_{oil\text{-}C}$ $kg^{-1}{}_{soil}$. The amended soils were placed in pre-cleaned amber glass jars, sealed with perforated aluminium foil and allowed to acclimatise in the dark at 21 ± 1 °C until 150 d, after which samples were analysed as described in the following sections. MBS solution (2 ml per 500 g of soil) was added to

all soils every 4 weeks in order to maintain high microbial viability. Composition of the MBS solution has been described elsewhere.[23]

2.3. Microbial activity and biomass-C determination

Microbial respiratory activity within the diesel-amended soil was quantified by monitoring CO_2 respiration rate in an aerobic closed static system in a MicroOxymax respirometer (Multiple-sensor CO_2/O_2 10 Chamber System, Columbus). Triplicate subsamples (20 g) were weighed into 250-ml Schott bottles and fitted to the MicroOxymax channels to measure background (basal) and substrate-induced respiration (SIR) respiration. The basal samples were treated similarly to the SIR samples expect that glucose (1.08 mg C g^{-1}) was not added. The initial maximum SIR rate (2–4 h) was used to estimate the "active" glucose-responsive biomass-C.[24] Metabolic quotient (qCO_2) was calculated as the ratio of basal respiration to biomass-C.[25]

2.4. Enumeration of B[a]P-degrading bacteria numbers

After 150-d acclimation, B[a]P-degrading bacteria in soils were quantified by measuring colony forming units (CFUs) following standard microbiological procedures from serial dilutions of 10^{-1} to 10^{-3}.[26] Soil (2 g) was extracted with quarter strength Ringer's solution in a 1:10; 1 ml of the aliquot was serially diluted with Ringer's solution. The resultant solution (0.1 ml) was spread on agar plates impregnated with B[a]P (5 mg l^{-1}) as sole source of carbon and incubated at 25 ± 1 °C; distinct colonies were counted after 12–14 d.

148 *2.5. Partitioning of ^{14}C-B[a]P in soils*

149 Following acclimation, the amended soils were spiked with $^{12}C/^{14}C$-B[a]P standards
150 prepared in toluene to deliver 10 mg B[a]P kg^{-1} with an associated ^{14}C-activity of ~83
151 kBq kg^{-1}.[22] To minimise the effect of toluene, the B[a]P standard was added to ¼ of the
152 soil and allowed to vent before adding the remaining soil. The spiked soils were
153 sterilised by a series of autoclaving–re-incubation three times over three alternate days.
154 The effectiveness of this procedure was previously determined by plating out 1 g of
155 soil on nutrient agar and potato-dextrose gar and incubating at 25 °C for 7–10 d; the
156 method was chosen because it has the least impact on soil particle stability as compared
157 to others.[27] The spiked soil subsamples (1.25 g; n = 3) were place in Oak Ridge
158 Teflon® centrifuge tubes and 5 ml of sterile deionised water added; tubes were then
159 incubated upright in the dark at 21 ± 1°C for 5 d with gentle agitation (40 rpm). This
160 allowed for extensive equilibration and partitioning of B[a]P molecules between
161 aqueous and solid phases.[11] An aliquot (1 ml) of sodium azide (10 g l^{-1}) was added to
162 maintain sterility during this period.[28]

163 Aqueous extraction was carried out by adding 25 ml 0.01 M $CaCl_2$ solution to each
164 centrifuge tubes and incubated in an end-over-end position on a flatbed rotary shaker
165 (150 rpm) for 22–24 h. Following centrifugation (3000 x g for 1 h), 5 ml was
166 withdrawn and mixed with 15 ml liquid scintillation cocktails in a 20-ml vial. The rest
167 of the supernatant was carefully decanted, soil drained and 25 ml aqueous solution of
168 50 mM HP-β-CD was added to the tube. The processes of shake-extraction,
169 centrifugation and sampling of supernatant were carried out three times (once after 22
170 h and the other two times after 2 h shake-extractions); the spent supernatant was
171 discarded and replaced with fresh HP-β-CD solution after each centrifugation and
172 sampling. The resulting pellets were re-suspended in 25 ml of distilled water for 10

173 min and centrifuged to ensure the complete removal of remaining HP-β-CD solution.
174 Thereafter, the ^{14}C-activity in all supernatants was summed up. Further extractions of
175 the HP-β-CD-extracted pellets with alkaline solution to quantify humic/fulvic acids
176 fractions were carried out (up to 5 times in the Holme soil and 3 times in the other
177 soils) until clear pale-yellow supernatants were obtained. The first extraction was with
178 30 ml $Na_4P_2O_7$:NaOH (1:20) and subsequently with 20 ml $Na_4P_2O_7$:NaOH (1:1). The
179 ^{14}C-activity from all extracted samples was quantified by liquid scintillation counting
180 (LSC) (Camberra Packard Tri Carb 2300TR, Camberra Packard, UK) using standard
181 calibration and quench correction techniques and appropriate protocols.
182 The extracted soil pellets were allowed to dry under fume hood, weighed (*ca.* 1 g) into
183 cellulose combustion cones and combusted (3 min) with the aid of Combustaid® (200
184 µl) (Packard 307 Sample Oxidiser); these represented the ^{14}C-fractions strongly-bound
185 (solvent-nonextractable) to humin materials. The efficiency of the Sample Oxidiser
186 was determined to be >96%. The ^{14}C-activity trapped with Carbosorb-E® (10 ml) and
187 Permafluor-E® (10 ml) was quantified by LSC as previously described.
188
189 *2.6. Mineralisation of ^{14}C-B[a]P in amended soils*
190 The standard radiorespirometric assay as described by Reid, et al. [29], was used to
191 monitor ^{14}C-B[a]P mineralisation for a period of 30 d. The respirometric bottle
192 consisted of 10 ± 0.2 g soil with 30 ml of sterile MBS solution with or without 50 mM
193 HP-β-CD (referred to MBS-only and MBS+HP-β-CD system, respectively). In
194 addition, fresh diesel (500 mg kg^{-1}) was applied as co-substrate to a set of microcosms
195 to produce MBS+diesel system. The $^{12/14}$C-B[a]P standard was delivered in 5 µl
196 toluene (per respirometer) as 10 mg ^{12}C-B[a]P kg^{-1} with an associated ^{14}C-activity of
197 ~83 kBq kg^{-1}. The respirometers were incubated on an orbital shaker (Janke and

198 Kunkel, IKA®-Labortechnik KS250, Germany) at 100 rpm under controlled laboratory
199 conditions (temperature 21 ± 1 °C; relative humidity 45%). During microbial
200 catabolism of ^{14}C-B[a]P, the $^{14}CO_2$ trapped with 1 M NaOH (1 ml) in a 7-ml vial was
201 mixed with 5 ml scintillation cocktail and quantified by LSC. The cumulative extents
202 (%), maximum rates (% d^{-1}) and lag phases (d, time before extent of $^{14}CO_2$ exceeds
203 5%) of were calculated from the mineralisation data.
204
205 *2.7. Statistical analysis*
206 Analyses of variance (ANOVA) followed by Holm-Sidak test, where there was
207 statistical significance ($P < 0.05$), was performed using SigmaStat ver. 3.5 (SPSS Inc.).
208 Paired *t*-tests with IBM SPSS 19 were carried out to compare between MBS-only and
209 MBS+HP-β-CD system or MBS+diesel system.
210
211 **3. Results**
212 *3.1. Soil physicochemical and microbiological indices*
213 The soils differed widely in their particle size profiles, SOM and clay contents, as well
214 as the available nutrient profiles (Table 1). For example, SOM and clay contents were
215 negligible in the predominantly sandy Antarctic soil but were 27% and 42%,
216 respectively, in the silt-clay Holme soil. Microbiological indices of unamended and
217 amended soils after 150 d acclimation are presented in Table 2. In unamended soils,
218 basal and SIR rates varied between soil types, being lowest in the Antarctic soil and
219 highest in the Holme soil, reflecting the organic carbon contents in the soils. In most of
220 the amended soils, basal respiration was comparable to their respective unamended
221 samples. However, SIR was significantly higher ($P < 0.05$) in the amended Holme and
222 Thurnham soils compared to their unamended samples. Microbial biomass-C was

223 lowest in the Antarctic soil and highest in the Holme soil. Except for the Antarctic soil,
224 microbial biomass-C contents were significantly ($P < 0.05$) higher and qCO_2 were
225 lower in the other amended soils relative to their unamended controls. The CFUs of
226 culturable B[a]P-degrading bacteria varied with soil type and were significantly ($P <$
227 0.05) higher in the amended than in unamended soils.
228
229 *3.2. Partitioning of ^{14}C-B[a]P in soils*
230 The influence of soil physicochemical characteristics as well as the effect of diesel
231 amendment on ^{14}C-B[a]P partitioning was assessed in the four soils (Table 3). Overall,
232 the partitioning of ^{14}C-B[a]P in the soil matrices was strongly influenced by their
233 texture and SOM contents, but diesel amendment had negligible effects. The amounts
234 of ^{14}C-B[a]P that partitioned into aqueous phase were not strongly influenced by SOM
235 contents (F-value = 4.635; $P = 0.053$). The HP-β-CD-extractable (bioaccessible) and
236 humic/fulvic acids-associated fractions of ^{14}C-B[a]P correlated strongly with SOM
237 contents (HP-β-CD: Holme < Kellet = Thurnham < Antarctic; F-value = 599.605; $P <$
238 0.001; humic/fulvic acids: Antarctic < Thurnham < Kellet < Holme; F-value = 64.061;
239 $P < 0.001$). Except for the Antarctic soil, relatively comparable amounts of ^{14}C-B[a]P
240 were associated with the humins of the other soils (Antarctic < Kellet = Holme =
241 Thurnham; F-value = 127.868; $P < 0.001$). Compared to their respective unamended
242 and 500 $mg_{oil\text{-}C}\ kg^{-1}{}_{soil}$ soils, relatively more ^{14}C-B[a]P partitioned to aqueous phase in
243 the 5000 $mg_{oil\text{-}C}\ kg^{-1}{}_{soil}$ soils (F-value = 6.846; $P < 0.05$). However, the HP-β-CD-
244 extractable and the humic/fulvic acids- or humin-associated fractions were not affected
245 by diesel amendment ($P > 0.05$). The solubility enhancement factor (SEF) defined as
246 the extent to which the presence of HP-β-CD increased the apparent aqueous solubility
247 of B[a]P ranged from 13 to 136 depending on soil type and soil treatments. It was

248 relatively lowest in the Holme soil with the highest SOM and in the 5000 $mg_{oil\text{-}C}\ kg^{-1}_{soil}$
249 soils than in the 500 $mg_{oil\text{-}C}\ kg^{-1}_{soil}$ or unamended soils.
250
251 *3.3. Mineralisation of ^{14}C-B[a]P in soils*
252 The relative contributions of HP-β-CD-enhanced solubilisation and diesel-enhanced
253 catabolic activity on ^{14}C-B[a]P mineralisation were measured in amended soils (Figure
254 1; Table 4). In addition, the influence of diesel as co-solvent or inducing substrate for
255 ^{14}C-B[a]P mineralisation was determined. The indices assessed were the lag phases,
256 fastest rates and extents of ^{14}C-B[a]P mineralisation. Overall, diesel amendment
257 significantly ($P < 0.05$) enhanced B[a]P mineralisation in all of the soils, though the
258 extents of ^{14}C-B[a]P mineralisation did not relate to any of the soil physicochemical
259 properties. Addition of fresh diesel facilitated greater ^{14}C-B[a]P mineralisation in soils
260 with established catabolic activity. The presence of HP-β-CD as solubility-enhancer
261 resulted in significant ($P < 0.05$) decreases to the extents of ^{14}C-B[a]P mineralisation in
262 most of the soils.
263
264 *3.3.1. Influence of diesel concentration on ^{14}C-B[a]P mineralisation*
265 Mineralisation of ^{14}C-B[a]P in the unamended and amended soils is displayed
266 graphically in Figure 1. Overall, the extents of ^{14}C-B[a]P mineralisation were low
267 (<5% of the added ^{14}C-B[a]P) in all unamended soils with very long lag phases (>30
268 d). Generally, there were significantly ($P < 0.05$) shorter lag phases in the amended
269 soils compared to their respective unamended controls (Table 4). Moreover, except for
270 the Nether-Kellet soil, significantly ($P < 0.05$) shorter lag phases were obtained in the
271 5000 $mg_{oil\text{-}C}\ kg^{-1}_{soil}$ soils compared to the respective 500 $mg_{oil\text{-}C}\ kg^{-1}_{soil}$ soils. Both
272 maximum rates and extents of ^{14}C-B[a]P mineralisation were significantly ($P < 0.05$)

higher in all 5000 $mg_{oil\text{-}C}\ kg^{-1}_{soil}$ soils compared to their respective unamended and 500 $mg_{oil\text{-}C}\ kg^{-1}_{soil}$ soils. For instance, collectively, exposure to 500 $mg_{oil\text{-}C}\ kg^{-1}_{soil}$ of diesel increased the overall extent of B[a]P mineralisation by factors of 2.45–27.64 and exposure to 5000 $mg_{oil\text{-}C}\ kg^{-1}_{soil}$ of diesel by factors of 4.67–44.13.

There are no strong correlations between the indices of ^{14}C-B[a]P mineralisation and any of soil physicochemical characteristics or the CFUs of B[a]P degraders in the soils. The low numbers of B[a]P degraders ($<10^4$ CFUs g^{-1}) reflected the inherently low catabolic potentials for B[a]P in the unamended soils. However, while diesel amendment increased the populations of B[a]P-degrading microorganisms, these increases did not correlate with the extents of ^{14}C-B[a]P mineralisation in the amended soils. For example, the ^{14}C-B[a]P catabolic activity was enhanced the most in the 5000 $mg_{oil\text{-}C}\ kg^{-1}_{soil}$ Nether-Kellet and the least in the 500 $mg_{oil\text{-}C}\ kg^{-1}_{soil}$ Antarctic soil, though both soils have comparable numbers of B[a]P degraders.

Mineralisation of ^{14}C-B[a]P in amended soils which were additionally amended with diesel is displayed graphically also in Figure 1. In general, the addition of fresh diesel (0.05% w/w) significantly ($P < 0.05$) increased maximum rates and extents of ^{14}C-B[a]P mineralisation in all previously amended soils, though it had variable effects on lag phases (Table 4). For example, following additional amendment with diesel, the lag phase was reduced from 18.92 to 11.36 d in the 500 $mg_{oil\text{-}C}\ kg^{-1}_{soil}$ sample but was extended from 11.37 to 18.57 d in the 5000 $mg_{oil\text{-}C}\ kg^{-1}_{soil}$ sample of the Antarctic soil. Eventually, however, additional amendment produced significantly ($P < 0.05$) higher extents of ^{14}C-B[a]P mineralisation in both amended Antarctic soils. Presence of fresh diesel increased the extents of ^{14}C-B[a]P mineralisation by almost 4-fold in the 500 $mg_{oil\text{-}C}\ kg^{-1}_{soil}$ sample and by about 2-fold in the 5000 $mg_{oil\text{-}C}\ kg^{-1}_{soil}$ sample compared to samples of the Antarctic soil not receiving additional diesel. Collectively for all the

298 soils, acclimation to diesel increased the extents of B[a]P mineralisation by factors of
299 2.45–44.13 while the additional amendment with fresh diesel further increased the
300 extents by factors of 6.71–71.06, as compared to the unamended soils.
301
302 *3.3.2. Influence of HP-β-CD-enhanced solubilisation on ^{14}C-B[a]P mineralisation*
303 The addition of HP-β-CD to enhance ^{14}C-B[a]P solubility and thus increase its
304 bioaccessibility and biodegradation was evaluated by comparing the MBS-only and
305 MBS+HP-β-CD systems (Table 4). Overall, addition of HP-β-CD appeared to have
306 negative effects on ^{14}C-B[a]P mineralisation. For example, presence of HP-β-CD
307 resulted in longer lag phases, reduced maximum rates and significantly ($P < 0.05$)
308 lower extents of ^{14}C-B[a]P mineralisation in all soils except the amended Thurnham
309 soils. Collectively for unamended, 500 $mg_{oil\text{-}C}$ kg^{-1}_{soil} and 5000 $mg_{oil\text{-}C}$ kg^{-1}_{soil} soils, the
310 presence of HP-β-CD impacted on the extents of B[a]P mineralisation by factors of
311 0.04–0.90 in the Antarctic, 0.56–2.09 in the Nether-Kellet, 0.59–1.74 in the Holme,
312 and 0.82–1.89 in the Thurnham soils, respectively.
313 Comparing the three systems (MBS-only, MBS+HP-β-CD and MBS+diesel)
314 investigated, generally the lag phases were longer, the maximum rates and the extents
315 of mineralisation lower in the MBS+HP-β-CD system compared to the other two
316 systems. For example, for the 500 $mg_{oil\text{-}C}$ kg^{-1}_{soil} Antarctic soil, the lag phases (18.92,
317 >30 and 11.36 d), the maximum rates (0.53, 0.24 and 2.10 % d^{-1}) and, the extents (9.62,
318 1.26 and 36.53 %) in the MBS-only, MBS+HP-β-CD and MBS+diesel systems,
319 respectively, were significantly ($P < 0.05$) different. Further, for all soils, while the lag
320 phases were fairly comparable, the maximum rates and the overall extents of
321 mineralisation were significantly ($P < 0.05$) higher in the MBS+diesel system than in
322 the other two systems. For the 500 $mg_{oil\text{-}C}$ kg^{-1}_{soil} Nether-Kellet and the 5000 $mg_{oil\text{-}C}$ kg^{-}

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320 phases were fairly comparable, the maximum rates and the overall extents of
321 mineralisation were significantly ($P < 0.05$) higher in the MBS+diesel system than in
322 the other two systems. For the 500 $mg_{oil\text{-}C}$ kg^{-1}_{soil} Nether-Kellet and the 5000 $mg_{oil\text{-}C}$ kg^{-}

323 $^{1}_{soil}$ Thurnham soils, the lag phases in the MBS-only, MBS+HP-β-CD and MBS+diesel
324 systems were similar (2.49, 1.82 and 2.40 d, respectively), but the maximum rates
325 (3.80, 5.21 and 14.99 % d^{-1}, respectively) and, the extents (28.43, 53.43 and 71.29 %,
326 respectively) were significantly ($P < 0.05$) different.
327
328 **4. Discussion**
329 *4.1. Effects of HP-β-CD-enhanced solubilisation and diesel-enhanced catabolic*
330 *activity on B[a]P biodegradation*
331 The results of B[a]P aqueous solubility and dissolution from soil matrices are
332 consistent with previous findings that B[a]P has very low bioaccessibility, and that the
333 adsorptive characteristics (SOM and clay contents) of soil influence its dissolution rate
334 from soil matrices.[30] The low bioaccessibility of PAHs in soils results from a
335 combination of low aqueous solubility and/or high sorption to lipophilic materials.[31]
336 The quantity and quality (i.e. degree of condensation) of SOM are known to influence
337 the pattern and extent of sorption of HOCs including PAHs to soil.[32] The results from
338 B[a]P partitioning to fulvic/humic acid and humin components of SOM supported this.
339 Further, this study is in agreement with other reports that have demonstrated increase in
340 the apparent solubility of PAHs and other HOCs in the presence of HP-β-CD or other
341 cyclodextrins.[19]. Cyclodextrins increase the concentration of HOCs in the aqueous
342 phase by solubilisation and decreased interactions with soil mineral and organic
343 fractions.[16, 33-35]
344 HP-β-CD-enhanced solubilisation of PAHs, such as naphthalene, phenanthrene and
345 pyrene, ordinarily improves the rate of microbial uptake with resultant increase in the
346 extent of mineralisation.[12, 13, 16] However, data from this study indicate that this is not
347 true for B[a]P. A number of other investigators have made similar observation.[18, 36]

Cuypers, et al. [36] have reported that the presence of HP-β-CD did not stimulate cometabolic degradation of poorly degradable 5–6-ring PAHs including B[a]P in two naturally contaminated sediments by an active microbial consortium. The authors suggested PAH degradation could have been inhibited as a result of a decreased free aqueous PAH concentration or as a result of preferential HP-β-CD biodegradation, which could have caused nutrient deficiency or the development of a microbial community unfit for the degradation of PAHs. Because HP-β-CD enhanced the ability of *Sphingomonas paucimobilis* strain EPA 505 to mineralise fluoranthene in a study by Mueller, et al. [37] but failed to promote B[a]P mineralisation in their own study, Ye, et al. [18] suggested that increasing the aqueous solubility of a PAH results in an increased biodegradation, mainly if the hydrocarbon is a growth substrate.

A remarkable finding of this study was that the presence of HP-β-CD did not only fail to promote B[a]P mineralisation, it actually negatively affected the different aspects of B[a]P biodegradation in three of the four soils investigated. This phenomenon could be related to the physical-chemical effect of the nature and stability of the inclusion complexes formed between HP-β-CD and B[a]P[38-40] as well as to the physiological effects of instantaneous bulk desorption of relatively high B[a]P concentration into the aqueous phase on the B[a]P-degrading microorganisms. Semple, et al. [41] has defined "*bioavailable compound* as that which is freely available to cross an organism's cellular membrane from the medium the organism inhabits at a given time… and, *bioaccessible compound* as that which is available to cross an organism's cellular membrane from the environment, if the organism has access to the chemical." The main difference in the definitions is the physical constrain imposed in time and/or space. In the light of the definitions and the results of this present study, it is postulated that HP-β-CD enhances B[a]P solubilisation (i.e. increases bioaccessibility); however,

373 the nature of the HP-β-CD–B[a]P inclusion complexation imposes physical constrains
374 on the actual bioavailability of B[a]P to cellular uptake and the subsequent
375 mineralisation.
376 Another plausible explanation of the observed significantly lower mineralisation of
377 B[a]P in the MBS+HP-β-CD compared to the MBS-only system is the effects of
378 increased toxicity due to high concentration of complexed B[a]P molecules
379 instantaneously desorbed and/or to the greater solubility of its rather more-harmful
380 metabolites, which could inhibit enzyme activities or suppress growth or even cause
381 death of susceptible organisms, resulting in changes to the B[a]P-degrading microbial
382 community. In addition, and as previously suggested[36], faster depletion of nutrients as a
383 result of either the preferential degradation of HP-β-CD or its simultaneous
384 degradation with B[a]P could lead to the observed lower mineralisation of B[a]P. This
385 latter reason seems less probable in this present study since, in the MBS+diesel system,
386 diesel also served as primary substrate for the cometabolic degradation of B[a]P and
387 resulted in the highest extents of mineralisation of all three systems investigated. The
388 results from this current study further corroborate the authors' previous work which
389 demonstrated that increasing the concentration of HP-β-CD reduced rather than
390 increased B[a]P mineralisation in a soil despite significantly enhancing the apparent
391 aqueous solubility of the contaminant (unpublished data).
392 Acclimation to diesel not only led to significantly ($P < 0.05$) reduced lag phases and
393 faster rates of ^{14}C-B[a]P mineralisation, but also increased the overall extents of
394 mineralisation in all soils. The data are comparable to those of other investigators.[42-44]
395 For example, in the five soils collected from an abandoned coal tar refinery in the study
396 by Grosser, et al. [42], the overall extents of mineralisation ranged from 25 to 70% for
397 pyrene and, from <1 to 40% for benz[a]anthracene after 64 d. Similar to the

398 observations reported in this current study, Grosser, et al. [42] noted that the soils with
399 higher hexane-extractable hydrocarbon contents consistently showed more rapid initial
400 rates and higher extents of mineralisation, and that the extents of mineralisation by
401 indigenous soil microflora appeared to be dependent more on the chemical
402 characteristics of the soil and less on soil total biomass and/or activity. Comparing
403 between the soil types, the differences in the various aspects of mineralisation appeared
404 to relate, to a larger degree, to soil biotic than abiotic properties. This agrees with the
405 finding by Macleod and Semple [45] who observed significant difference in the rate of
406 development of pyrene catabolic activity in two soils with similar physicochemical
407 properties. Measurable pyrene mineralisation (extent ≥5% of added ^{14}C-pyrene) was
408 observed in the pasture soil after only 8 weeks following amendment with 100 mg
409 pyrene kg^{-1}, but it took 76 weeks for such to occur in woodland soil.
410 The results further suggested that in addition to the enrichment of indigenous microbial
411 catabolic potentials, certain components of diesel acted as primary substrates to support
412 B[a]P co-mineralisation. Complete degradation of B[a]P by microbial consortia has
413 been demonstrated mainly in the presence of certain substrates acting as cometabolic
414 inducers.[44, 46, 47] More so, diesel, like other NAPLs, has been thought to act both as a
415 discrete co-sorbent for PAHs and/or to competitively displace PAHs, thereby reducing
416 their rate of sorption to SOM.[32, 48, 49] Overall, data from this study emphasised that the
417 low biodegradability of B[a]P often observed in many pristine and contaminated soils
418 is more likely due to the greater effects of low catabolic potentials of microorganisms
419 and/or absence of suitable primary substrates, and to a lesser extent on bioavailability
420 limitations caused by either soil abiotic characteristics or the PAH physicochemical
421 properties.
422

423 *4.2. Relevance of findings*

424 A major implication of the findings of this study is that, in practice, it may be futile or,
425 in certain circumstances, detrimental to try and improve B[a]P solubilisation in soils
426 lacking robust degradative ability to withstand and/or mineralise the increased B[a]P
427 aqueous concentration. For instance, unlike other LMW-PAHs, B[a]P degradation is
428 more complicated and often involved the accumulation of a number of characteristic
429 metabolites, which probably are more recalcitrant and exhibit greater genotoxic
430 properties than the parent compound.[1, 3] Evidence abound that a diol epoxide derivative
431 of B[a]P is the ultimate carcinogenic metabolite and remarkably, this compound is far
432 more soluble that the parent B[a]P.[50-53] In an event where B[a]P is metabolised to its
433 mutagenic and/or carcinogenic intermediates without further mineralisation,
434 particularly in the presence of HP-β-CD, may have far-reaching environmental impacts
435 on soil biota and heighten concern for public health safety. Sverdrup, et al. [54] has
436 demonstrated that the ability of B[a]P to exhibit narcotic effect on soil-dwelling
437 springtail *Folsomia fimetaria* was only limited by its water solubility. If HP-β-CD–
438 B[a]P complexes are very stable, as predicted by the stabilisation constants of
439 complexes HP-β-CD formed with other similar organic compounds[38, 40], its use in
440 bioremediation of B[a]P-contaminated soils and sediments may present greater risks to
441 underground aquifers and increased toxicity to other environmental receptors, which
442 may result in additional cost for bioremediation.

443 In conclusion, it has been demonstrated in this study that, diesel-enhanced catabolic
444 activity made greater contribution to B[a]P mineralisation than HP-β-CD-enhanced
445 solubilisation. Data presented here give some insights into the practicality of the effect
446 of HP-β-CD-enhanced solubilisation on bioaccessibility and biodegradation of B[a]P in
447 contaminated soils. To the authors' knowledge, this is the first report evaluating the

448 influence of soil physical–chemical characteristics on HP-β-CD-enhanced
449 biodegradation of PAH. Furthermore, this study provides data to support the claims
450 that extensive mineralisation of B[a]P requires the presence of suitable co-substrates.
451 Currently, there is limited knowledge about the nature and stability of the HP-β-CD–
452 B[a]P inclusion complexes and how these are affected by environmental and edaphic
453 factors, as well as the presence of co-contaminants. Therefore, there is need for further
454 investigation of the variables influencing the physical, chemical and biological
455 interactions of cyclodextrin–PAH–soil under complex contaminant mixture systems.
456

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631 **Tables**

632

633 Table 1: Physicochemical properties of soils.

Properties	Soils			
	Antarctic	Nether-Kellet	Holme	Thurnham
Classification	Typical sandy	Typical brown-earth	Earthy oligo-fibrous peat	Typical humic alluvial gley
Texture	Sandy	Loam	Silty clay	Clay loam
Sand (60–2000 μm) %	94.69	55.39	10.45	38.77
Silt (2–60 μm) %	5.31	26.77	47.37	34.44
Clay (<2 μm) %	<0.01	17.84	42.18	27.79
Organic C (%)	0.25	2.99	19.50	3.48
C:N ratio	<1	12	15	13
Soil organic matter (%)*	<1	9.33	27.15	10.25
pH (dH_2O)	6.25	5.44	7.50	6.93
Available P (mg kg^{-1})	3	15	34	42
Available K (mg kg^{-1})	1027	239	297	752
Available Mg (mg kg^{-1})	393	2503	1523	2249
Available Ca (mg kg^{-1})	876	1857	1626	888
2-ring PAHs (ng g^{-1})	nd#	30.91	6.53	nd
3-ring PAHs (ng g^{-1})	2.95	37.06	55.22	30.01
4-ring PAHs (ng g^{-1})	2.48	13.16	6.43	3.23
5–6-ring PAHs (ng g^{-1})	2.44	22.12	23.47	9.05
∑16 USEPA PAHs (ng g^{-1})	7.87	103.25	91.65	42.29
Benzo[a]pyrene (ng g^{-1})	Nd	1.41	nd	0.42

634 * Loss on ignition
635 # not detected

636 Table 2: Some microbiological indices in the amended soils.[§]

Soil type	Treatment ($mg\ kg^{-1}$)	Respiratory Responses ($\mu g\ g^{-1}\ h^{-1}$) Basal[†]	SIR[ǂ]	Biomass-C ($\mu g\ g^{-1}$ soil DW)[#]	qCO_2 ($\mu g\ CO_2$-C mg^{-1} biomass h^{-1})	B[a]P-degraders ($x\ 10^4$ CFUs g^{-1})
Antarctic	0	$0.14 \pm 0.04^{a‡}$	6.86 ± 0.85^{a}	104.04 ± 34.82^{a}	1.37 ± 0.79^{a}	<0.1 (<0.1)
	500	1.44 ± 0.02^{b}	6.25 ± 0.53^{a}	125.20 ± 10.70^{a}	11.63 ± 0.86^{b}	12.3 (4.00)
	5000	1.57 ± 0.11^{b}	6.67 ± 0.10^{a}	109.85 ± 8.02^{a}	14.56 ± 2.05^{b}	8.57 (2.67)
Nether-Kellet	0	3.53 ± 0.03^{a}	160.52 ± 6.64^{a}	537.80 ± 6.68^{a}	6.56 ± 0.14^{a}	0.56 (0.16)
	500	2.89 ± 0.12^{a}	94.02 ± 16.90^{b}	797.68 ± 96.95^{b}	3.73 ± 0.60^{b}	12.8 (3.47)
	5000	1.81 ± 0.28^{a}	87.10 ± 17.32^{b}	748.04 ± 3.14^{b}	2.41 ± 0.36^{b}	14.2 (5.02)
Holme	0	7.35 ± 0.25^{a}	180.57 ± 13.89^{a}	624.70 ± 31.55^{a}	11.85 ± 1.00^{a}	0.42 (0.55)
	500	3.62 ± 0.06^{b}	298.01 ± 15.89^{b}	1342.44 ± 12.71^{b}	2.70 ± 0.07^{b}	10.3 (3.60)
	5000	7.28 ± 0.22^{a}	830.20 ± 174.77^{c}	3105.71 ± 153.40^{c}	2.36 ± 0.19^{b}	16.6 (2.47)
Thurnham	0	6.05 ± 0.75^{a}	146.82 ± 26.71^{a}	518.33 ± 61.22^{a}	12.18 ± 2.88^{a}	0.80 (0.32)
	500	2.58 ± 0.24^{b}	442.07 ± 46.36^{b}	1532.45 ± 16.00^{b}	1.68 ± 0.14^{c}	18.2 (3.74)
	5000	8.26 ± 0.41^{a}	490.36 ± 63.04^{b}	1809.00 ± 79.04^{b}	4.57 ± 0.02^{b}	14.7 (5.88)

637 § Amended soils were analysed after 150 d of acclimation.
638 † Values (mean ± SEM) are average of 2-hourly continuously measurements for 24 h in triplicate samples.
639 ǂ SIR is the maximum glucose-induced respiration within 24 h.
640 # "Active" glucose-responsive biomass-C
641 ‡ Different lower-case letters down the column are statistically significant (P <0.05).
642

643 Table 3: Partitioning of added ^{14}C-B[a]P in the diesel-amended soils.[§]

Soil type	Soil treatment (mg kg^{-1})	Aqueous (%)	HP-β-CD (%)	FA + HA (%)	Humins (%)	Total recovery (%)	SEF
Antarctic	0	0.52 ± 0.11[†]	70.74 ± 9.84	19.30 ± 2.83	6.16 ± 1.73	96.71 ± 12.20	136
	500	0.72 ± 0.14	73.49 ± 6.13	24.22 ± 3.44	3.25 ± 1.15	101.68 ± 9.96	102
	5000	0.81 ± 0.04	66.58 ± 1.17	22.18 ± 1.40	6.44 ± 2.33	96.02 ± 2.83	82
Nether-Kellet	0	1.07 ± 0.09	20.52 ± 4.10	32.41 ± 3.80	42.52 ± 7.48	96.51 ± 8.75	19
	500	0.86 ± 0.06	22.35 ± 1.82	36.94 ± 4.82	43.52 ± 3.35	103.66 ± 7.47	26
	5000	1.47 ± 0.43	23.05 ± 2.74	31.12 ± 2.48	45.20 ± 2.07	100.84 ± 5.36	16
Holme	0	0.63 ± 0.48	13.03 ± 0.49	42.33 ± 9.19	45.47 ± 4.57	101.46 ± 13.45	21
	500	0.77 ± 0.11	15.86 ± 1.68	43.83 ± 3.70	41.36 ± 3.09	101.60 ± 5.96	21
	5000	0.94 ± 0.03	12.12 ± 0.94	39.25 ± 2.17	43.01 ± 0.41	95.32 ± 3.36	13
Thurnham	0	0.61 ± 0.02	19.80 ± 4.28	23.14 ± 1.62	53.43 ± 2.96	96.97 ± 6.65	32
	500	1.06 ± 0.31	24.81 ± 0.50	26.87 ± 2.71	42.50 ± 1.51	95.25 ± 3.99	23
	5000	1.29 ± 0.47	20.90 ± 0.93	27.32 ± 3.69	55.43 ± 2.40	104.94 ± 5.56	16

644 § Amended soils were analysed after 150-d of acclimation.
645 † Values are means ± SEM of three independent measurements.
646 FA + HA: Fulvic/humic acids
647

Table 4: Mineralisation of ^{14}C-B[a]P (10 mg kg^{-1}) in diesel-amended soils.

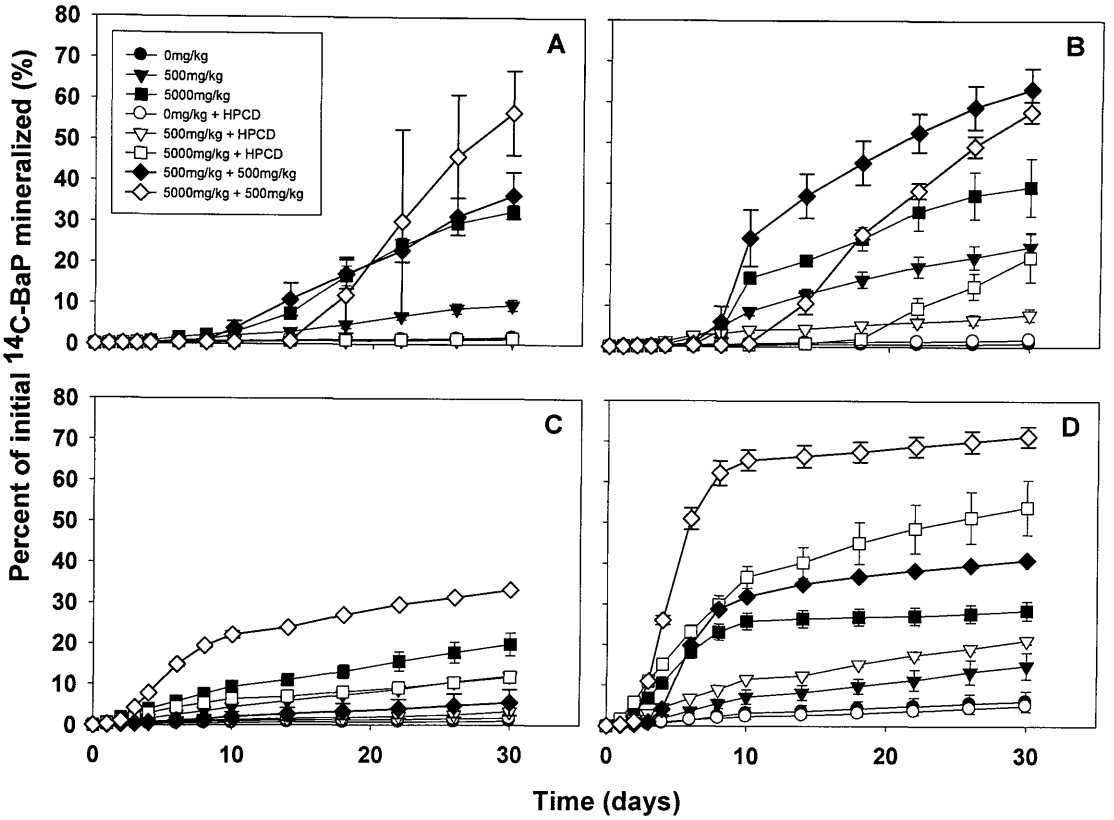
Soil type	Treatment (mg kg^{-1})	Lag phases (d)			Maximum rates (% d^{-1})			Cumulative extent (%)		
		MBS-only	MBS+HPCD	MBS+diesel	MBS-only	MBS+HPCD	MBS+diesel	MBS-only	MBS+HPCD	MBS+diesel
Antarctic	0	>30^{c‡}	>30^{a}		0.16 ± 0.02^{a}	0.19 ± 0.03^{a}		1.75 ± 0.76^{a}	1.58 ± 0.39^{a}	
	500	18.92 ± 2.54^{b}	>30^{a*}	11.36 ± 1.43$^{a\#}$	0.53 ± 0.36^{a}	0.24 ± 0.04^{a}	2.10 ± 0.45$^{a\#}$	9.62 ± 1.34^{b}	1.26 ± 0.10^{a*}	36.53 ± 5.71$^{a\#}$
	5000	11.37 ± 0.91^{a}	>30^{a*}	18.57 ± 3.13$^{b\#}$	2.31 ± 0.62^{b}	0.15 ± 0.03^{a*}	4.51 ± 3.32^{a}	32.56 ± 1.68^{c}	1.48 ± 0.10^{a*}	56.75 ± 10.30$^{b\#}$
Nether-Kellet	0	>30^{b}	>30^{c}		0.14 ± 0.01^{a}	0.15 ± 0.01^{a}		0.89 ± 0.08^{a}	1.86 ± 0.65^{a}	
	500	8.39 ± 0.24^{a}	13.32 ± 0.62^{a*}	7.63 ± 0.66^{a}	1.77 ± 0.36^{b}	0.76 ± 0.15^{a}	10.31 ± 1.53$^{a\#}$	24.60 ± 3.59^{b}	7.94 ± 1.48^{b*}	63.24 ± 5.12$^{a\#}$
	5000	11.96 ± 3.11^{a}	19.94 ± 0.68^{b*}	11.91 ± 0.55^{b}	6.44 ± 0.01^{c}	1.90 ± 0.65^{a*}	4.24 ± 0.38$^{b\#}$	39.28 ± 7.08^{b}	22.01 ± 5.95^{c}	57.66 ± 2.56$^{a\#}$
Holme	0	>30^{c}	>30^{b}		0.14 ± 0.02^{a}	0.17 ± 0.06^{a}		0.97 ± 0.09^{a}	1.69 ± 0.47^{a}	
	500	>11.71^{¶b}	>30^{b*}	19.51 ± 0.52$^{a\#}$	0.28 ± 0.11^{b}	0.25 ± 0.04^{a}	0.75 ± 0.04$^{a\#}$	5.50 ± 3.21^{b}	3.25 ± 0.55^{a*}	11.68 ± 0.85$^{a\#}$
	5000	5.14 ± 0.18^{a}	7.82 ± 0.89^{a*}	3.23 ± 0.07$^{b\#}$	1.17 ± 0.08^{c}	0.94 ± 0.11^{b}	3.69 ± 0.08$^{b\#}$	19.80 ± 2.80^{c}	11.75 ± 1.50^{b*}	33.18 ± 0.16$^{b\#}$
Thurnham	0	>8.90^{¶b}	30.00 ± 0.00^{c*}		0.47 ± 0.31^{a}	0.37 ± 0.03^{a}		6.09 ± 2.48^{a}	4.99 ± 0.16^{a}	
	500	8.12 ± 1.88^{b}	4.43 ± 0.23^{b*}	4.11 ± 0.01$^{a\#}$	0.96 ± 0.15^{a}	1.45 ± 0.22^{b}	7.86 ± 0.10$^{a\#}$	14.91 ± 3.23^{b}	21.12 ± 0.85^{b*}	40.89 ± 0.11$^{a\#}$
	5000	2.49 ± 0.18^{a}	1.82 ± 0.05^{a*}	2.40 ± 0.02^{b}	3.80 ± 0.67^{b}	5.21 ± 0.36^{c}	14.99 ± 1.31$^{b\#}$	28.43 ± 2.31^{c}	53.87 ± 6.74^{c*}	71.29 ± 2.51$^{b\#}$

‡ Different lower-case letters down the column for each "soil type" are statistically different ($P < 0.05$).
* Asterisk indicated that mineralisation indices in HP-β-CD-supplemented microcosms are statistically different from those of unsupplemented microcosms ($P < 0.05$).
# Ash tag indicated that mineralisation indices in diesel-supplemented microcosms are statistically different from those of unsupplemented microcosms ($P < 0.05$).
¶Only one of the triplicate samples reached >5% mineralisation before the end of the experiment.

654 **Figure**

655

656



657
658

659

660 Figure 1: Mineralisation of ^{14}C-B[a]P (10 mg kg^{-1}) in (A) Antarctic (B) Nether-Kellet

661 (C) Holme and (D) Thurnham soils. Legends: MBS-only systems: 0 (●), 500 (▼) and

662 5000 (■) $mg_{oil\text{-}C}$ kg^{-1}_{soil}. MBS+HP-β-CD (50 mM) systems: 0 (○), 500 (▽) and 5000

663 (□) $mg_{oil\text{-}C}$ kg^{-1}_{soil}. MBS+diesel systems: 500 + 500 (♦) and 5000 + 500 (◊) $mg_{oil\text{-}C}$ kg^{-}

664 $^{1}_{soil}$. The symbols represent means and the bars where visible are standard error the

665 means (n = 3).

666

Paper VIII

There is no relationship between endpoint mineralisation rate and amount of benzo[a]pyrene residues that remained bioaccessible in soil slurries

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10 **Abstract**

11 The extent to which benzo[a]pyrene biodegradation is influenced by bioavailability and
12 biodegradability limitations was investigated in four soils with different sorptive
13 characteristics. To achieve varying benzo[a]pyrene degradative ability, the soils were
14 pre-exposed to different diesel concentrations. Mineralisation of ^{14}C-benzo[a]pyrene
15 was measured in soil slurries until it plateaued; subsequently, the putative bioaccessible
16 ^{14}C-residues left in soil slurries (measured using HP-β-CD extraction) and the ^{14}C-
17 residues partitioned to humic/fulvic acids and humin components of soil were
18 quantified. After plateauing, the steady rates of mineralisation (0.02–3.43% d^{-1}) were
19 mostly low and independent of the amounts of the bioaccessible ^{14}C-residues, which
20 were highly variable (4–67%) between soil treatment conditions. Partitioning of ^{14}C-
21 residues to humic/fulvic acids and humin components differed markedly with soil
22 types. Overall, results indicated that, unlike most other PAHs that may serve as
23 microbial growth substrates, the termination of benzo[a]pyrene mineralisation was
24 influenced more by factors constraining microbial degradative activity and to lesser
25 extent by the soil sorption-limiting chemical bioavailability.

26

27 **Capsule:**

28 Factors promoting or limiting microbial degradative activity are the major drivers of
29 B[a]P mineralisation in soils.

30

31 **Keywords**: *Benzo[a]pyrene; Bioaccessibility; Bioavailability; Diesel oil;*
32 *Mineralisation; PAH; Soil*

33

1. Introduction

Benzo[a]pyrene is a five-ring polycyclic aromatic hydrocarbon (PAH) which has been extensively investigated because of its ecological and ecotoxicological significance (Hu et al. 2007; Juhasz & Naidu 2000). Benzo[a]pyrene and the products of its biotransformation present serious risk to human and wildlife health in that it has high propensity to bio-accumulate in living tissues and exhibit chronic genotoxicity including carcinogenic and immuno-toxic effects (Hu et al. 2012). Due to its physicochemical properties, such as low water solubility (3.8 $\mu g\ l^{-1}$) and a high hydrophobicity (log K_{ow} 6.06), benzo[a]pyrene, like other PAHs, tends to readily adsorb to soil organic carbon (SOC) or diffuse into soil micropores by numerous physical and chemical interactions (White et al. 1999; Xing & Pignatello 1997). Additionally, benzo[a]pyrene is highly recalcitrant to microbial oxidation because it is unsuitable as a labile source of carbon and energy, making it to persist in the soil (Juhasz & Naidu 2000; Kanaly & Harayama 2000). Broadly, these physicochemical and biological properties of benzo[a]pyrene reflect on its bioaccessibility and/or biodegradability in soil.

Bioaccessibility is thought to be the governing factor controlling the rate of biodegradation of PAHs in soil (Semple et al. 2006; Yang et al. 2009b). However, several studies have evidenced a lack of correlation between the bioaccessibility of benzo[a]pyrene and its biodegradation in soil (Huesemann et al. 2004; Juhasz et al. 2005b). Further, soil abiotic and biotic characteristics are known to a play key role in the environmental fate of PAHs by influencing their bioaccessibility and/or biodegradability. It has been evidenced that soil sorptive matrices (including SOC and clay materials, black carbon, kerogens) significantly decrease bioaccessibility and limite the rates of PAH biodegradation over time (Ehlers & Loibner 2006; Ortega-

59 Calvo et al. 1997; Ortega-Calvo & Saiz-Jimenez 1998; Rhodes et al. 2008; Weissenfels
60 et al. 1992). Specifically, there are sufficient citations in the literature to support the
61 fact that SOC and clay affect benzo[a]pyrene bioaccessibility (e.g., Stroo et al. 2000).
62 Presently, however, the effects of soil types on benzo[a]pyrene biodegradability remain
63 poorly understood; there is limited information to allow for comparison of the effects
64 of soil abiotic and biotic characteristics on benzo[a]pyrene biodegradability in different
65 soils. In particular, studies that compared benzo[a]pyrene biodegradation in disparate
66 soils under similar pollution conditions are rare. Meanwhile, such studies are important
67 because the successful implementation of *in situ* bioremediation of PAH-contaminated
68 sites is contingent upon a good understanding of how the varieties of soil biotic and
69 abiotic characteristics affect the development and evolution of degradative capability,
70 especially towards higher-molecular-weight (HMW)-PAHs like benzo[a]pyrene.
71 Further, it is well-accepted that mineralisation rate tend to dramatically reduce or stop
72 when the bioaccessible fraction of a hydrophobic contaminant is depleted in soil
73 solution (Semple et al. 2006; Yang et al. 2009b). However, it is yet unclear whether
74 this applies also to compounds that ordinarily do not serve as growth substrates, or that
75 their mineralisation depends on cometabolic degradation with other growth substrates.
76 Benzo[a]pyrene is a typical example of a non-growth substrate, requiring the presence
77 of co-metabolite(s) for its biodegradation (Kanaly & Watanabe 2004). Therefore, this
78 study aimed to assess the extent to which benzo[a]pyrene mineralisation is influenced
79 by factors limiting bioaccessibility and/or biodegradability in soils with differing
80 abiotic characteristics, and that their benzo[a]pyrene degradative potentials have been
81 altered.
82

2. Materials and methods

2.1. Chemicals

Non-labelled benzo[a]pyrene and [7,-^{14}C]benzo[a]pyrene (13.8 mCi mmol^{-1}, > 96% pure) were supplied by Amersham Corp., USA. Goldstar multipurpose liquid scintillation fluid and 7-ml and 20-ml glass scintillation vials were supplied by Meridian, UK. Sodium hydroxide and tetrasodium orthophosphate were from Merck, UK. Carbosorb-E® and Permafluor-E® sample oxidizer cocktails were obtained from Perkin-Elmer Life Sciences, USA and Combustaid® from Canberra Packard, UK. Diesel fuel was obtained at a local BP fuel station in Lancaster, UK. Hydroxypropyl-β-cyclodextrin (purity >96%) was obtained from Acros Organics, USA. All other solvents and chemicals used were of reagent grade or better.

2.2. Soil treatment

The physicochemical and microbiological characteristics of the four pristine soils used are presented in Table 1; methods for their determination have been described elsewhere (Okere et al. 2012; Rhodes et al. 2010). Prior to soil treatment with diesel, sieved soil samples were equilibrated in the dark at 21 ± 1 °C for 10 d to allow microbial activity to stabilise. Soil treatment with diesel (in 5 ml acetone kg^{-1}) was carried out following the single-step spiking/rehydrating (to 60% WHC) procedure described by Doick et al. (2003) to deliver the oil at concentrations of 0.05 and 0.5% w/w. For the pre-exposure microcosms, soil subsamples (200 g) were placed in amber jars covered with perforated aluminium foil and then acclimated in the dark at 21 ± 1 °C for 30 d. For optimal microbial activity, fortnightly, spiked soils were briefly exposed to air and thoroughly mixed with spatula to prevent anoxic conditions. Loss of

107 moisture was checked by weighing and where necessary, moisture content adjusted
108 with deionized water.
109
110 *2.3. Mineralisation of ^{14}C-benzo[a]pyrene in soil slurry*
111 Indigenous catabolism of benzo[a]pyrene was investigated in the following soil slurry
112 systems: (i) unamended soils, (ii) 0.05%–freshly-spiked soils, (iii) 0.5%–freshly-spiked
113 soils, (iv) 0.05%–pre-exposed soils, and (v) 0.5%–pre-exposed soils. The radio-
114 respirometric assay described by Reid et al. (2001) was used to measure mineralisation
115 rate of 10 mg benzo[a]pyrene kg^{-1} (^{14}C-activity 83 kBq kg^{-1}) in the spiked soils (10 ±
116 0.2 g). All respirometers were incubated in the dark (21 ± 1 °C; 100 rpm) until
117 mineralisation plateaued. Periodically, the $^{14}CO_2$ trapped with 1 M NaOH (1 ml) was
118 quantified by liquid scintillation counting (LSC, Canberra Packard Tri Carb 2300TR,
119 UK). Mineralisation was terminated after 30 d by adding 1 ml NaN_3 (10 g l^{-1}) to poison
120 the microbial degraders (Kirk et al. 2004). Prior to this, aliquots (1 ml) of soil slurries
121 were collected for microbial enumeration using the standard spread plate technique
122 (Lorch et al. 1995). Previous studies using this respirometric assay showed that where
123 rapid mineralisation of PAH was feasible, the rate had considerably declined by 12–15
124 d (Doick et al. 2006; Semple et al. 2006). More so, it has also been shown in soil slurry
125 tests that beyond 25 d PAH mineralisation rates do not further increase significantly
126 (Latawiec & Reid 2009). Abiotic microcosms (heat-sterilised by repeated autoclaving–
127 re-incubation for 3 alternate days) were used to monitor loss through abiotic loss. The
128 effectiveness of this sterilisation technique was initially verified by plating out 1 g of
129 sterilised soil on nutrient agar and potato-dextrose gar and incubating at 25 °C for 7–10
130 d. The method was chosen because it has the least impact on soil particle stability as
131 compared to others (Getenga et al. 2004). Blank respirometers were included to

132 monitor background radioactivity in the soils. All microcosms were set up in
133 triplicates.
134
135 *2.4. Extraction of ^{14}C-residues in soil slurry after mineralisation has plateaued*
136 A scheme of sequential extractions and sample oxidations were used to assess the
137 distributions of ^{14}C-residues after mineralisation was terminated. The procedure was
138 also carried out on the heat-sterilised samples to evaluate the effect of soil
139 physicochemical characteristics on benzo[a]pyrene solubility and sorption in soil.
140 *(i) Aqueous extraction*: content of the soil slurry in each respirometer was carefully
141 transferred quantitatively into a Teflon-lined centrifuge tube (50 ml). Samples were
142 centrifuged twice (3600 x *g*; 30 min) on a Beckman Centaur 2 centrifuge. The
143 supernatant was carefully filtered, the volume determined, and an aliquot (3 ml)
144 sampled into a 20 ml vial containing 15 ml scintillation fluid. The ^{14}C-activity was
145 quantified by LSC.
146 *(ii) Hydroxypropyl-β-cyclodextrin (HP-β-CD) extraction*: After separating the liquid
147 phase from soil-solid materials, the filter cakes were re-suspended in 30 ml HP-β-CD
148 solution (50 mM) and end-to-end shake-extracted for 22 h. Following centrifugation
149 which was repeated twice with fresh HP-β-CD solution, the supernatants were
150 combined, 5 ml aliquot withdrawn and the ^{14}C-activity quantified by LSC.
151 *(iii) Fulvic acid/humic acid (HA/FA) extraction*: The ^{14}C-residues bound to SOM were
152 further fractionated into humic/fulvic acids- and humin-associated residues. The post-
153 HP-β-CD extracted-pellets were allowed to dry under vacuum in the fume-hood for 24
154 h. The pellets were then re-suspended first in 30 ml of 0.1 M $Na_4P_2O_7.12H_2O$:0.1 M
155 NaOH (1:20) base solution and end-to-end shake-extracted for 24 h; the headspace in
156 centrifuge tube was purged with N_2 gas before extraction. After centrifugation, aliquot

157 (1 ml) of the supernatant was sampled into a 20 ml vial containing 15 ml scintillation
158 fluid; and the remainder was carefully decanted and volume determined. Fresh 30 ml
159 base solution of 0.1 M $Na_4P_2O_7.12H_2O$:0.1 M NaOH (1:1) was added and the
160 extraction repeated for 1 h. The suspension was centrifuged (3000 *g*, 30 min) and
161 supernatant sampled (1 ml) and the reminder carefully decanted each time; this process
162 was repeated several times until the supernatant became pale-yellow to colourless
163 (Macleod & Semple 2003). No further fractionation of ^{14}C-residues in the humic/fulvic
164 acids was carried out; it is acknowledged that ^{14}C-residues in the base extracts may not
165 be totally attributed to those only bound to HA/FA, since the HM-bound fractions may
166 desorb in the process of extraction. Thus, the alkaline extracts may include the HA/FA-
167 bound solutes and any solute desorbed from HM during the extraction, but the ^{14}C-
168 residues in HM matrices would actually represent the highly resistant fraction bound in
169 the soil (von Lützow et al. 2007; White et al. 1999). The ^{14}C-activity was quantified
170 using the appropriate protocols as calibrated on the LSC.
171 *(iv) Sample oxidation of extracted soil pellet*
172 The dried extracted soil pellet (*ca.* 1 g) was combusted (3 min) using a Packard model
173 307 sample oxidiser (Canberra Packard, UK) to quantify ^{14}C-residues remaining after
174 the series of sequential extractions. The ^{14}C-activity combusted to $^{14}CO_2$ was trapped in
175 10 ml Carbosorb-E® and 10 ml Permafluor® used as scintillant. Quantification was also
176 by LSC. Prior to combustion, the efficiency of the machine was determined (>95% at
177 any time).
178
179 *2.5. Definition of terms, presentation of data and statistical analysis*
180 All mineralisation data were initially corrected for soil background radioactivity. The
181 rate ^{14}C-benzo[a]pyrene mineralisation was monitored as percent $^{14}CO_2$ evolved per

182 day of total added ^{14}C-benzo[a]pyrene. Mineralisation had plateaued before the assay
183 was terminated. Statistical analysis of the three data points taken between day 22 and
184 day 30 of respirometric assays indicated that mineralisation rates were steady during
185 this period. Hence, the rate at day 30 is presented here and is referred to in the Results
186 and Discussion sections as "steady mineralisation rate" or "steady rate." Wherever it
187 appears in the manuscript, "HP-β-CD-extractable" or "potentially bioaccessible ^{14}C-
188 residues" mean ^{14}C-residues extracted with aqueous-only and HP-β-CD solutions. This
189 expression is used to simplify ensuing discussion and should not be overtly interpreted
190 as or confused with such related terms as bioavailability and bioaccessibility (see
191 Semple et al. 2004), which are defined with respect to a parent chemical and not
192 necessarily including their metabolites. In this manuscript, for clarity purposes, "^{14}C-
193 residues" putatively include the untransformed parent ^{14}C-benzo[a]pyrene, its ^{14}C-
194 metabolites and new ^{14}C-materials formed during the oxidation process that were left in
195 the soil slurries.
196 Collectively, ^{14}C-activity recovered in all soils was very high and ranged 85–112% of
197 the added ^{14}C-benzo[a]pyrene. For the presentation of the ^{14}C-residues data the
198 following mass balance was used: Total ^{14}C-residues = (^{14}C-residues in aqueous phase
199 + ^{14}C-residues extracted with HP-β-CD solution + ^{14}C-residues extracted as HA/FA-
200 associated with base solution + ^{14}C-residues as HM-associated in combusted soil
201 pellet).
202 All statistical analyses were performed using SigmaStat for Windows (Ver. 3.05, SPSS
203 Inc.).
204

205 **3. Results**

206 The selected soils and the treatments used in this study were to reflect the influence of
207 both soil adsorptive materials (e.g., organic carbon and clay components) and varying
208 enhancement conditions on bioaccessibility and biodegradability of benzo[a]pyrene.
209 Following a period of 30 d incubation, extents of ^{14}C-benzo[a]pyrene mineralisation
210 varied widely between soil types and treatment conditions (Figure 1). Overall,
211 mineralisation was significantly enhanced in the freshly-spiked and pre-exposed soils
212 compared to their respective unamended soils. The distributions of ^{14}C-residues left in
213 the soil slurry after mineralisation was terminated are showed in Figure 1. The data
214 presented as stacked bars were normalised to the aggregates of ^{14}C-residues recovered
215 in aqueous and HP-β-CD solutions as well as in humic/fulvic acids and humin
216 components of soil. The actual values are shown on the top of the stacked bars. There
217 was no quantifiable mineralisation to $^{14}CO_2$ in the autoclaved soil samples, indicating
218 the process of sterilisation was sufficient to attenuate the benzo[a]pyrene-degrading
219 microorganisms in soil. The distributions of ^{14}C-benzo[a]pyrene in these sterile soils
220 reflected the influence of soil type on bioaccessibility. Over 70% of the added ^{14}C-
221 benzo[a]pyrene was HP-β-CD-extractable in soil A (<1% organic carbon, undetectable
222 clay and 95% sand contents) and about 80% of the added ^{14}C-benzo[a]pyrene was
223 retained in the humic/fulvic acids and humin components of soil C (20% organic
224 carbon, 42% clay and 10% sand).

225 In the biologically active microcosms, the distributions of ^{14}C-residues also reflected
226 the influence of soil types. The HP-β-CD-extractable ^{14}C-residues (F = 10.529, P <
227 0.001) and ^{14}C-residues associated with humic/fulvic acids (F = 6.164, P = 0.005) or
228 humin components (F = 3.824, P = 0.039) were strongly correlated to the soil
229 adsorptive materials. However, the level of degradative activity, as determined by

mineralisation extents, appeared to have effects which did not reflect a particular pattern across the different soil types. For example, in soil A the ^{14}C-residues remaining as potentially bioaccessible (HP-β-CD-extractable) were lower in the microcosms with higher degradative activity. In soil B or C, the potentially bioaccessible ^{14}C-residues were similar for a soil type despite that degradative activities were significant different. The ratios of ^{14}C-residues in the humic/fulvic acids to the humin component were fairly related to the level of degradative activity in microcosms of soils B but such pattern was not reflected in soil C. For soil D, the potentially bioaccessible ^{14}C-residues were higher in microcosms with higher degradative activity.

The steady rates of mineralisation varied substantially between different soil types and treatments (Table 2). For example, the steady rates were 0.22, 1.46, 0.02, and 0.25% d^{-1} for unamended microcosm of soils A, B, C and D, respectively. In term of the diesel concentration in the freshly-spiked soils, steady rate was higher at lower concentration in soil A, and lower at higher concentration in soil B. In terms of pre-exposure time, steady rates were higher in the freshly-spiked soils C and D compared to their respective pre-exposed samples. Overall, this is indicative of variability in the responses of soil microorganisms to diesel. This shows that the kind of treatments applied to soil achieved the aim of obtaining samples of a soil with differing benzo[a]pyrene-degrading activity. There are no correlations or identifiable patterns between the steady rates of mineralisation and the amounts of ^{14}C-residues in the aqueous phase (Figure 2) or that were potentially bioaccessible influence in soil slurries (Figure 3). Except for samples of soil A, the amounts of ^{14}C-residues optimally extracted with HP-β-CD solution were not quite larger than those effectively remaining in aqueous solution of soil slurries for the other soils (Figures 2 and 3). For soil A,

255 larger amounts of ^{14}C-residues were further extracted with HP-β-CD due to its poor
256 adsorptive properties. From the data, it was estimated that *ca.* 117, 5, 170 and 17 d are
257 required for this ^{14}C-residues pool to be converted to $^{14}CO_2$ in unamended soils A, B, C
258 and D, respectively. This view is supported by the facts that the extent of
259 mineralisation was high in soil B and very low in soil C after 30 d incubation, and that
260 the amounts of ^{14}C-benzo[a]pyrene initially bioaccessible were significantly different
261 in soils A and D due to the difference in their characteristics. Meanwhile, in diesel-
262 treated soils B, C and D, *ca.* 3 to 11 d are required for complete conversion to $^{14}CO_2$.
263

264 **4. Discussion**

265 Respirometric assays similar to the one used in this study are commonly employed to
266 monitor the microbial degradative activity in soil slurry systems, as they allow for the
267 complete distribution of the added ^{14}C-PAH and produce faster mineralisation
268 (Hatzinger & Alexander 1995; Ortega-Calvo et al. 1995; Reid et al. 2001; White et al.
269 1997). Further, it was demonstrated that this respirometric assay can be used to
270 estimate microbial availability of PAHs in soil (Semple et al. 2006). Unsurprising
271 though, extent of benzo[a]pyrene mineralisation did not to match the predicted
272 bioaccessible fraction using HP-β-CD extraction; previous studies have reported
273 overestimation of benzo[a]pyrene bioaccessibility by various non-exhaustive extraction
274 techniques (NEETs), including HP-β-CD extraction (Cuypers et al. 2000; Hawthorne
275 et al. 2001; Papadopoulos et al. 2007; Reid et al. 2000). It appears that the
276 bioaccessibility estimates for benzo[a]pyrene in soils only represent a fraction of the
277 compound that is potentially accessible to impact on ecological receptors and may not
278 necessarily reflect the fraction that is actually biodegradable (Naidu et al. 2008). The
279 low degradative potential for benzo[a]pyrene in most of the soils investigated may be

280 linked, in part, to the compound's refractory properties, and in part, to the low numbers
281 of benzo[a]pyrene degraders and very low background concentrations of the
282 contaminant in the soils (Bamforth & Singleton 2005; Seo et al. 2009). The
283 enhancement of benzo[a]pyrene biodegradation and mineralisation by diesel, as
284 evidenced in this study, has previously been linked to certain constituents of diesel
285 which promoted the growth of degrading populations and acted as co-substrates for
286 cometabolic degradation of benzo[a]pyrene (Kanaly et al. 2001; Kanaly & Watanabe
287 2004).
288 The wide range in the organic carbon (0.25 to 20%) and clay contents (undetectable to
289 42%) of the soils investigated provided a good basis for the comparison between the
290 effects of soil–PAH interactions on bioaccessibility and rates of mineralisation of
291 benzo[a]pyrene in soil slurries. Consistent with the finding of this study, strong
292 correlations between PAH bioaccessibility and soil organic matter and clay contents
293 have been reported by other investigators (Bielská et al. 2012; Rhodes et al. 2008;
294 White et al. 1999; Xing & Pignatello 1997; Yang et al. 2010). Studies have showed
295 both enhancing and inhibitory effects of organic carbon on biodegradation of PAHs in
296 soil, with the body of evidence leaning towards inhibition (Bogan & Sullivan 2003;
297 Liang et al. 2007; Manilal & Alexander 1991; Ortega-Calvo et al. 1997; Yang et al.
298 2009a). In the present study, apparently there was no indication that soil abiotic
299 characteristics influenced benzo[a]pyrene mineralisation. This is mainly a result of the
300 significant differences in the indigenous degradative ability for benzo[a]pyrene of the
301 soils investigated. Probably, in an experiment wherein the same degrader inoculum is
302 added to sterile samples of these soils, the outcomes might reflect the effect of soil
303 abiotic characteristics.

304 The rate of biodegradation of PAHs in soil is thought to be controlled by the
305 degradative activity of microorganisms and the mass transfer of a chemical to the
306 microorganisms (Bosma et al. 1997; Semple et al. 2003). Bosma et al. (1997) applied a
307 generic mathematical concept for bioavailability to the measured biotransformation
308 kinetics of organic compounds in soil slurries and in percolation column, and found
309 that mass transfer and not the intrinsic microbial activity was, in most cases, the critical
310 factor in bioremediation. An exception wherein intrinsic degradative activity has
311 greater influence than mass transfer is the biodegradation of non-growth substrate like
312 benzo[a]pyrene in soil slurries, as evidenced by the results from this present study.
313 Collectively, the data clearly demonstrated that mineralisation was independent of the
314 amounts of benzo[a]pyrene that was bioaccessible in soil slurries. As mineralisation
315 plateaued, the amounts of ^{14}C-residues that remained potentially accessible to
316 microorganisms were comparatively high enough to sustain faster rates than the steady
317 rates measured. In addition, the CFUs of benzo[a]pyrene-degrading bacteria were
318 observed to be higher after than before the respirometric assays were conducted (data
319 not shown). Hence, it is postulated that the potentially bioaccessible ^{14}C-residues were
320 in the form that was no longer microbially available and/or that the further
321 transformation ultimately to $^{14}CO_2$ had greatly slowed down because the increased
322 accumulation impacted negatively on the extant microbial community structure, which
323 resulted in reduced degradative activity. Time-course analysis of DGGE profiles during
324 diesel-enhanced mineralisation of benzo[a]pyrene has revealed changes in the
325 emergence and re-emergence of populations within the bacterial consortium (Kanaly et
326 al. 2000).
327 The results of this study further emphasize that it is not only critical that a compound
328 must be available in a labile form to the extant microorganisms, it also must be

329 inherently biodegradable or at least co-metabolisable by the competent organisms
330 (Semple et al. 2003) – conditions which are seldom met in the case of benzo[a]pyrene.
331 Like other PAHs, for the microbial degradation of benzo[a]pyrene to ensue, the
332 compound must first be physically available for mobilisation into the microbial cell
333 prior to its transformation by specialised enzymes (Semple et al. 2007). The *physical*
334 *availability* of a chemical to microbial cells has been described as *bioaccessibility*
335 (Semple et al. 2004). To differentiate this from the actual *biological availability* – often
336 the rate limiting phase – which arises when a compound passes through the biological
337 barrier to the site of biological response, another term was described as *bioavailability*
338 (Semple et al. 2004). Inadvertently, the terms bioaccessibility and bioavailability have
339 been used interchangeably in the literature, although bioavailability is thought to be
340 more difficult than bioaccessibility to measure chemically using current laboratory
341 techniques (Collins et al. 2013; Cui et al. 2013; Doick et al. 2006; Semple et al. 2007;
342 Stroo et al. 2000). In practical terms, bioavailability has been demonstrated to be
343 organism- and even species-specific (Bogan et al. 2003; Friedrich et al. 2000). In
344 effect, the significance of these concepts to the present study tends to explain why a
345 compound may not be bioavailable though it is potentially bioaccessible. Our results
346 are in conformity with a number of other studies (Cuypers et al. 2000; Huesemann et
347 al. 2004; Juhasz et al. 2005a) which reported little or no microbial degradation of high
348 molecular weight PAHs (≥5-ring compounds) during bioremediation even after
349 extensive incubation periods, although bioaccessibility assessment suggested that these
350 compounds were available for degradation. Collectively, the authors have attributed
351 this lack or low level of benzo[a]pyrene biodegradation to biological factors (e.g., high
352 activation energies, unfavourable Gibbs free energy, slow transport over cell
353 membrane, and the inability of microorganisms to grow on low aqueous

concentrations) (Bonten et al. 1999), rather than bioavailability issues (Cuypers et al. 2000; Huesemann et al. 2004; Juhasz et al. 2005a).

Several studies have evidenced that many of the metabolites from benzo[a]pyrene biotransformation are particularly more polar and they often exhibit greater genotoxic effects (Newbold & Brookes 1976; Routledge et al. 2001; Stroo et al. 2000; Vrabie et al. 2011). Thus, in situations where factors constraining microbial degradative activity and not substrate mass transfer limit the bioconversion of benzo[a]pyrene to innocuous products the eventual accumulation of polar metabolites will increase toxicity to sensitive ecological receptors. For example, increased bioaccessibility and risk of PAHs have been reported in a soil under-going large-scale bioremediation (Andersson et al. 2009). Responses of the CALUX AhR agonist and Comet genotoxicity bioassays, as well as chemical analysis indicated increased toxicity in soil to organisms after 274 d of treatment than in the untreated soil. This was attributed to the release of previously sorbed PAHs and possible metabolic formation of novel toxicants (Andersson et al. 2009). A recent study on field-contaminated soil from a former MGP site reported significant reductions in residual PAH levels, but increased toxicity and genotoxicity over the course of a 7-d treatment cycle in a slurry-phase bioreactor (Hu et al. 2012).

In summary, the results of this study indicate that factors constraining degradative activity have greater effect than bioavailability on the termination of benzo[a]pyrene mineralisation. This finding further highlights the need to incorporate the principles of bioaccessibility and bioavailability into the assessment of remedial measures for the clean-up of PAH-contaminated soils (Semple et al. 2004; Stroo et al. 2000). This is particularly important for contaminated sites carrying a large burden of the recalcitrant and carcinogenic benzo[a]pyrene without possessing either the requisite assemblage of

competent microorganisms for effective decontamination or strong adsorptive surfaces for entrapment of the contaminants by bound-residue formation.

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576 **Tables**

577

578 Table 1: Physicochemical and microbiological properties of soils.

Properties	Soils			
	A	B	C	D
Grid reference	Antarctica	SD491655	SD511775	SD447543
Texture	Sandy	Loam	Silty clay	Clay loam
pH (dH_2O)	7.90	5.44	7.50	6.93
Sand (60-2000 μm) %	94.69	55.39	10.45	38.77
Silt (2-60 μm) %	5.31	26.77	47.37	34.44
Clay (<2 μm) %	0	17.84	42.18	27.79
C:N ratio	<1	12	15	13
Soil organic matter (%)*	<1	9.33	27.15	10.25
Available P (mg kg^{-1})	3	15	34	42
Available K (mg kg^{-1})	1027	239	297	752
Available Mg (mg kg^{-1})	393	2503	1523	2249
Available Ca (mg kg^{-1})	876	1857	1626	888
Biomass-C (μg g^{-1})#	103	1362	2344	2040
Fungal/Bacterial biomass ratio	0.02	1.09	1.02	1.25
Total bacteria (10^5 CFU g^{-1})	1.16	498.0	2590	307
B[a]P degraders (10^3 CFU g^{-1})	0.21	5.20	1.30	6.20
qCO_2 (μg mg^{-1} biomass h^{-1})	1.07	5.22	3.86	4.72
Benzo[a]pyrene (ng g^{-1})	ND	1.41	ND	0.42
∑16 USEPA PAHs (ng g^{-1})	7.87	103.25	91.65	42.29
Soil classification	Typical sandy	Typical brown-earth	Earthy oligo-fibrous peat	Typical humic alluvial gley

579 * Loss on ignition
580 qCO_2: Metabolic quotient
581

582 Table 2: Steady rate (endpoint) of mineralisation in the soil slurries.

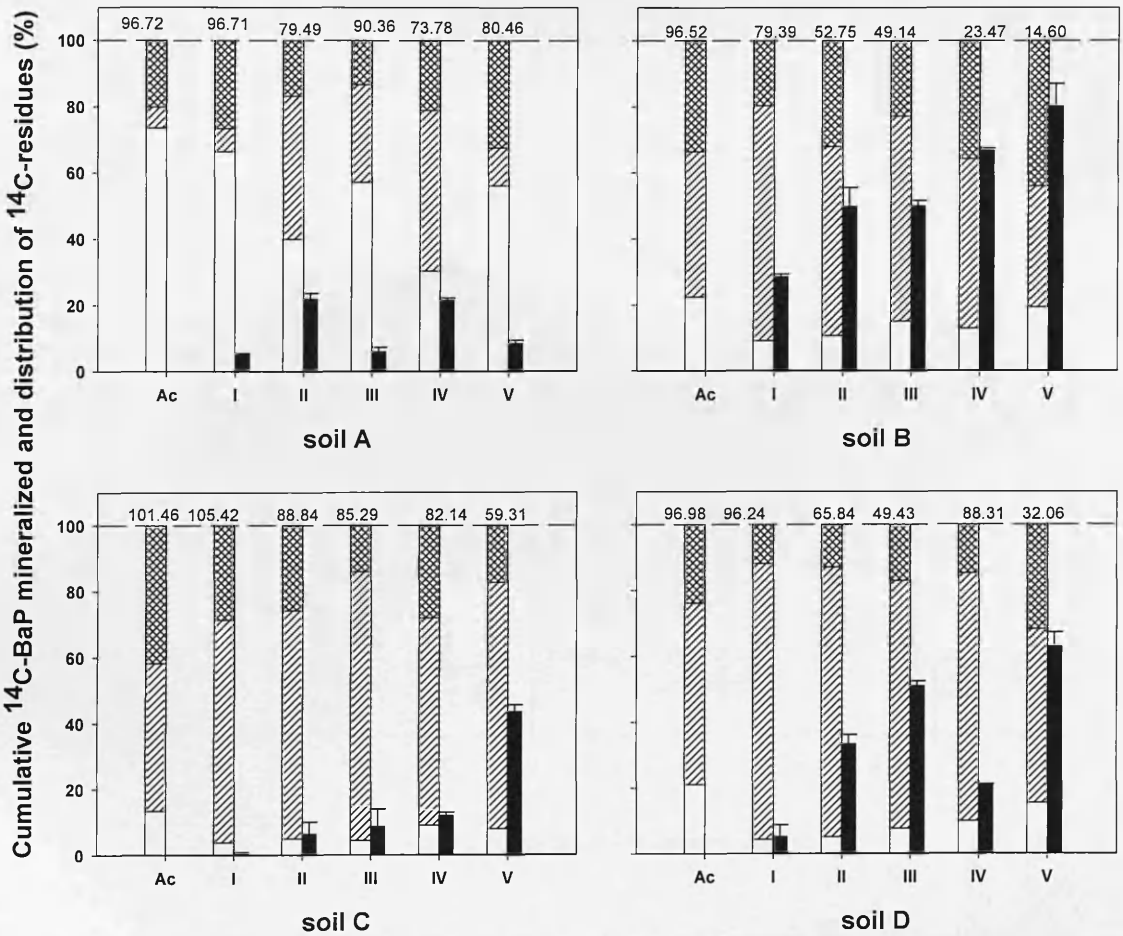
Soil treatment	Steady rate of mineralisation at day 30 (% d^{-1})			
	Soil A	Soil B	Soil C	Soil D
Unamended	0.22 ± 0.04	1.46 ± 0.12	0.02 ± 0.01	0.25 ± 0.18
0.05%–freshly-spiked	0.40 ± 0.07	0.76 ± 0.12	0.40 ± 0.29	1.08 ± 0.16
0.5%–freshly-spiked	0.15 ± 0.06	3.43 ± 1.06	1.06 ± 0.78	1.21 ± 0.18
0.05%–pre-exposed	0.47 ± 0.02	0.42 ± 0.16	0.22 ± 0.06	0.19 ± 0.18
0.5%–pre-exposed	0.15 ± 0.01	1.02 ± 0.28	0.41 ± 0.14	0.53 ± 0.09

583

584

585 **Figures**

586

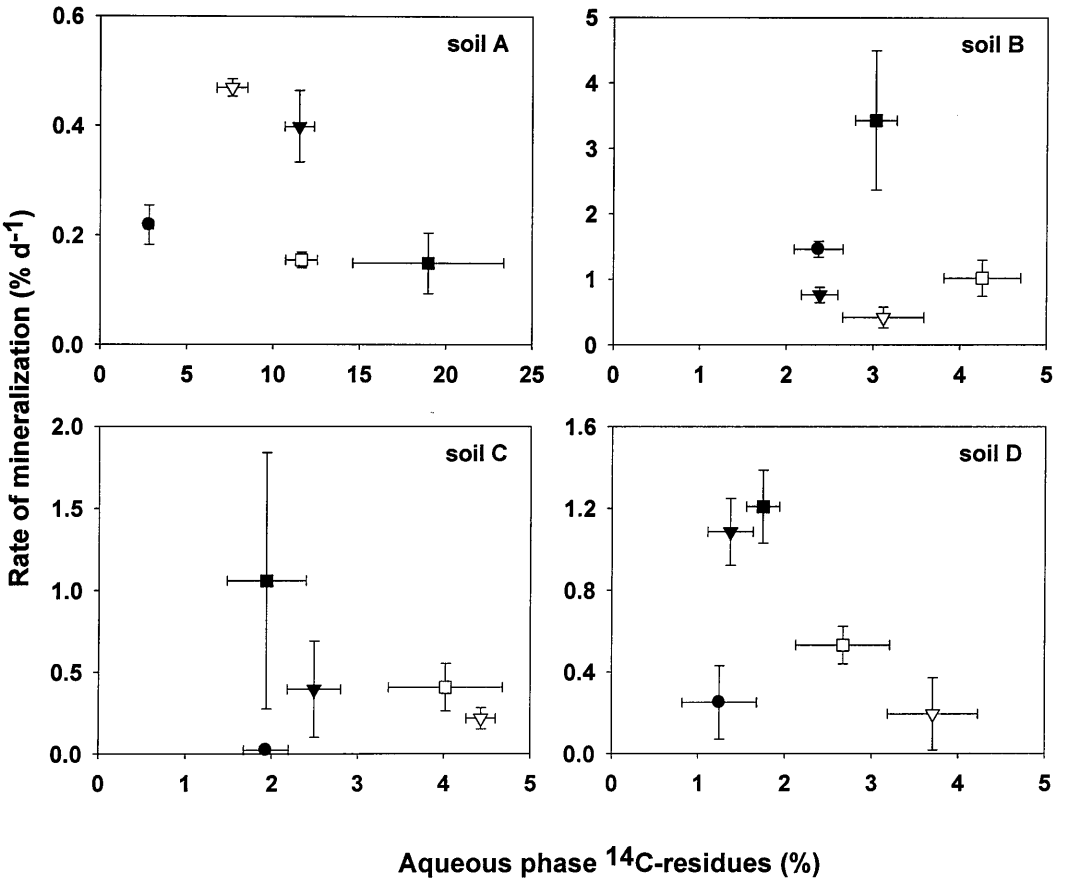


587
588

589 Figure 1: Cumulative ^{14}C-benzo[a]pyrene mineralised to $^{14}CO_2$ (black bar) and the
590 distributions of ^{14}C-residues (normalised to the recoverable ^{14}C-activity after
591 bioassays) as HP-β-CD-extractable (white); humic/fulvic-associated (diagonal-
592 hatched); and humin-associated (cross-hatched) in the soil slurries. Soils were
593 autoclaved (Ac), unamended (I), 0.05%–freshly-spiked (II) 0.05%–pre-exposed (III)
594 0.5%–freshly-spiked (IV), and 0.5%–pre-exposed (V). Values on the top of stacked
595 bars indicate percentage of the added ^{14}C-benzo[a]pyrene (10 mg kg^{-1}) recovered as
596 ^{14}C-residues.

597

598

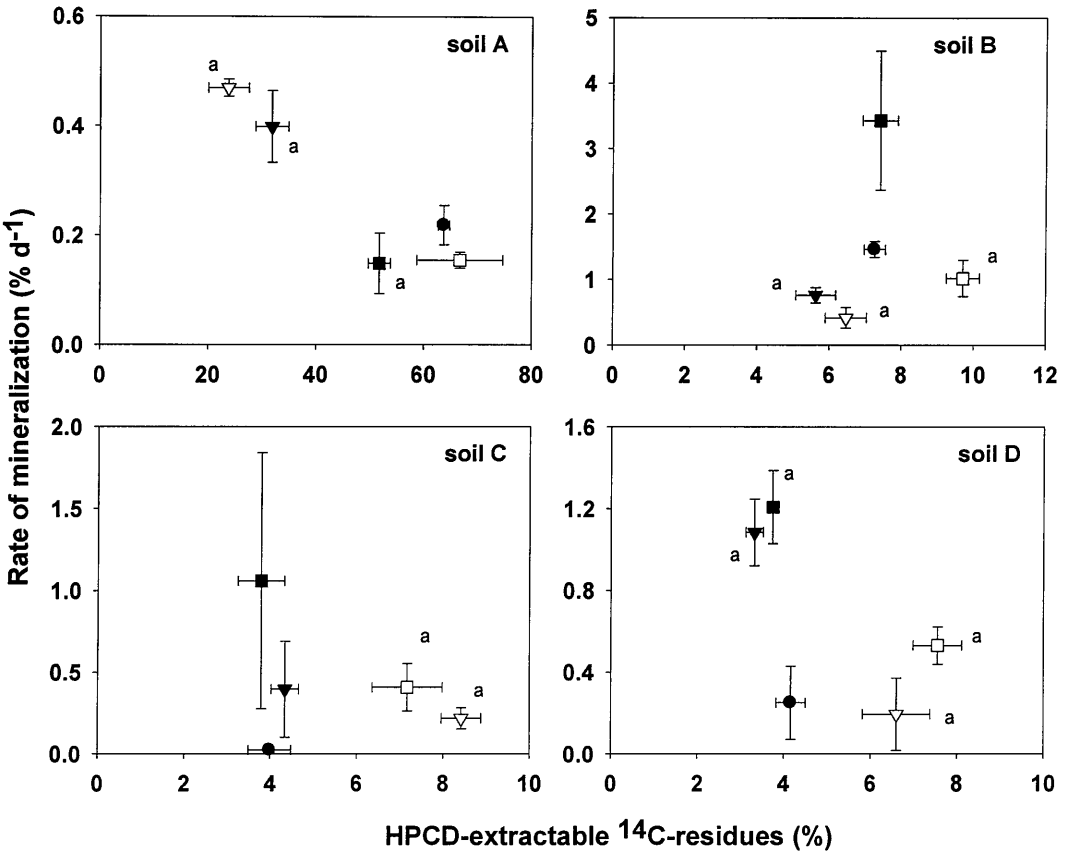


599
600

601 Figure 2: Relationship between steady rates of mineralisation and ^{14}C-residues in
602 aqueous phase after 30-d incubation. Soils were unamended (●) 0.05%–freshly-spiked
603 (▼), 0.5%–freshly-spiked (■) 0.05%–pre-exposed (▽), or 0.5%–pre-exposed (□).

604

605



606
607

608 Figure 3: Relationship between steady rates of mineralisation and HP-β-CD-extractable
609 ^{14}C-residues after 30-d incubation. Soils were unamended (●) or 0.05%–freshly-spiked
610 (▼), 0.5%–freshly-spiked (■) 0.05%–pre-exposed (▽), and 0.5%–pre-exposed (□).
611 Lower-case letter 'a' indicates that the maximum rate was significantly greater ($P <$
612 0.05) than the steady rate reached at the time mineralisation was terminated.

613

Paper IX

Biodegradability of naphthalene, phenanthrene and benzo[a]pyrene in diesel oil-contaminated soil after exposure to prescribed fire

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12 **Abstract**

13 In practice, due to certain site constraints, cost-effectiveness of alternative options
14 and/or regulatory requirements, prescribed fire may be permitted as a first-line
15 remediation option for large petroleum oil spills; however, the impact on the
16 degradative ability of indigenous soil microbial community has rarely been
17 investigated. Following short-term (1-, 30- and 60-d) and long-term (240-d) post-
18 treatments, the indigenous catabolism of ^{14}C-naphthalene, ^{14}C-phenanthrene or ^{14}C-
19 benzo[a]pyrene was monitored in diesel oil-amended soils (0, 0.05 or 0.5% w/w)
20 treated with or without prescribed fire (200–250 °C, 0.5 h). Diesel alone had marginal
21 effects on the catabolism of naphthalene or phenanthrene, but significantly enhanced
22 benzo[a]pyrene catabolism, depending on the initial oil level and time post-treatments.
23 Basically, soil-burning alone had negligible long-term effects on the catabolism of ^{14}C-
24 PAHs. The combined effect of diesel and soil-burning was dependent on the PAH
25 chemical structure–biodegradability; this being short-term for naphthalene and
26 phenanthrene and long-term for benzo[a]pyrene. In general, soil treatments had no
27 effects on the polarity of ^{14}C-residues while the effects on microbial abundance
28 (measured as CFUs) were short-term. It is suggested that the effects of prescribed fire
29 on the development of indigenous PAH catabolism in a diesel oil-amended soil, are
30 largely dependent on PAH chemical structure, the initial oil level and time post-
31 treatments.

32

33

34 **Keywords**: *Benzo[a]pyrene, Diesel oil, Naphthalene, Mineralisation, PAH,*
35 *Phenanthrene, Prescribed fire; Soil burning; Alternative remediation*

36

Capsule:

Effects of prescribed fire on the development of indigenous PAH catabolism in a diesel oil-amended soil, are largely dependent on the PAH chemical structure, the initial oil level and time post-treatments.

Highlights:

- ► *Effects of diesel oil (0.05 or 0.5%) and soil-burning on PAH catabolism investigated.*
- ► *Initial oil level has marginal effect on naphthalene or phenanthrene catabolism.*
- ► *Enhancement of B[a]P catabolism depends on initial oil level and time post-treatments.*
- ► *Soil-burning alone has marginal effect on catabolism of PAHs.*
- ► *PAH catabolism in burnt soil depends on initial oil level and time post-treatments.*
- ► *No effect on polarity of ^{14}C-residues; effects on microbial abundance short-termed.*

1. Introduction

Since the advent of the industrial revolution in the mid-18th century, and the later engineering improvements made to the internal combustion engine in early 20th century, there has been tremendous increase in the production, and usage of petroleum oil products as a principal source of energy to drive heavy machineries. Exploration and transport of crude petroleum and its refined oils, as well as the wide-scale generation and disposal of petroleum oil wastes represent an on-going concern for the sustainability of the natural environment. Large-scale spills of petroleum oils can significantly impact on vast expanses of sensitive ecosystems with enormous effects on wildlife and human society; severe damages to aquatic and terrestrial habitats, injuries and sometimes death of plants and animals have been reported, as reviewed by Aguilera et al. (2010).

A large collection of both laboratory-based and field-scale studies has evidenced a variety of effects, ranging from beneficial to detrimental, on the capacity of soil microbial community to resist and/or adapt to perturbations caused by wildfires or prescribed fires (Bååth et al., 1995; Boerner et al., 2000; Pietikäinen et al., 2000; Lindau et al., 2003; Zengel et al., 2003; Certini, 2005; Campbell et al., 2008; Gray and Dighton, 2009; Swallow et al., 2009; Rietl and Jackson, 2012; Chalbot et al., 2013). However, most of the studies available in the literature are primarily directed at the effects of prescribed fires as a natural resource and land-use management strategy with little attention being paid to the impacts as contaminated land remediation option. In practice, due to the peculiar limitations in wetlands and coastal marshes, such as accessibility constraints to sites, the prohibitive cost and/or ineffectiveness of alternative options to specific site conditions, as well as the regulatory requirements for immediate mandatory actions, *in situ* prescribed fire is often used as a first-line

79 remediation operation to remove a large portion the oil contaminants from the top soil
80 or sediment surface (Lin et al., 2002; Lindau et al., 2003; Zengel et al., 2003; Lin et al.,
81 2005). This operation may also be used in inland and upland environments to prevent
82 spreading of oil to sensitive sites or larger areas or reduce the generation of oily wastes,
83 especially where transportation or disposal options are limited (Zengel et al., 2003).
84 Much of the understanding of the impact of prescribed fires for oil spill remediation is
85 based on work in the open sea, wetlands and other coastal land environments, with
86 research efforts focussed toward general ecological function and structure including
87 species composition and density, above- and below-ground productivity, vegetation
88 and soil resiliency, soil physics and chemistry, soil residual oil, and organic matter
89 decomposition (Baustian et al., 2010). The impact of prescribed fires (and sometimes
90 accidental or deliberate act of sabotage) on the indigenous microbial community in
91 petroleum oil-contaminated upland environment has rarely been investigated (Zengel et
92 al., 2003), and to date, there is no study of the impact on degradative ability of the
93 indigenous soil microflora to catabolise the residual oil.
94 In petroleum oil-contaminated soils, although aliphatic hydrocarbons are dominant,
95 they are of lesser threat to human health than polycyclic aromatic hydrocarbons
96 (PAHs) because the latter group include compounds with toxic, carcinogenic and
97 mutagenic properties, in addition to the recalcitrant nature of their chemical structure
98 (Semple et al., 2003). Due to the potential ecological and ecotoxicological risk
99 associated with PAHs, as of January 2008 the US Environmental Protection Agency
100 (US EPA) has designated 28 PAHs as priority pollutants (Gan et al., 2009).
101 Biodegradation is a major mechanism for the removal of PAHs from the environment;
102 however, a number of studies has shown that the initial oil level affect the rate and
103 extent of biodegradation because at higher concentrations the contaminants usually

exhibit increased toxicity to microorganisms (Bogan et al., 2005; Swindell and Reid, 2007).

Therefore, the main aim of this present work was to evaluate the effects, individually and jointly, of diesel contamination (500 and 5000 mg kg^{-1}) and soil-burning (200–250 °C, 0.5 h), on the PAH degradative ability of the extant indigenous soil microorganisms after short-term (1–60 d) and long-term (240 d) post-treatments. The influence of the PAH chemical structure–biodegradability on these effects was also assessed.

2. Materials and methods

2.1. Materials

Unlabelled naphthalene, phenanthrene and benzo[a]pyrene (purity >99%, HPLC-grade), [7-^{14}C]naphthalene (specific activity = 55 mCi $mmol^{-1}$, radiochemical purity >99.6%) and [9-^{14}C]phenanthrene (55.7 mCi $mmol^{-1}$, >99%) were obtained from Sigma–Aldrich Co., UK, while [7-^{14}C]benzo[a]pyrene (13.8 mCi $mmol^{-1}$, >95%) was from Amersham Corp., USA. Goldstar multipurpose liquid scintillation fluid and 7-ml and 20-ml glass scintillation vials were supplied by Meridian (Epsdom, UK), Carbosorb-E® and Permafluor® were obtained from Perkin-Elmer Life Sciences, USA while sodium hydroxide was from Merck (UK). Diesel oil used in this experiment was a commercial grade no. 2 fuel obtained at a local BP fuel station in Lancaster, UK. The oil had total organic carbon content of 87%, and contained 86% diesel range organics (nC$_8$–nC$_{25}$) and 10% gasoline range organics (nC$_6$–nC$_{10}$), as determined by gas chromatography; naphthalene and phenanthrene but not benzo[a]pyrene were detected. A pristine sandy loam soil from an Ah-horizon of a Dystric Cambisol was collected from Myerscough Agricultural College (SD496402), Lancashire, UK. The percentages

129 of soil organic matter, sand, silt and clay are approximately 4.82, 55.63, 24.96 and
130 19.41, respectively. The soil has not been contaminated with PAHs from anthropogenic
131 source; ∑16 PAHs (30μg kg^{-1}): naphthalene (1); phenanthrene (18); but
132 benzo[a]pyrene was below the detection level. All other solvents used were from
133 Sigma–Aldrich Co., UK and of reagent grade or better.
134
135 *2.2. Soil treatment and incubation*
136 The soil was air-dried for 24–48 h, sieved (≤2 mm) and stored at 4 °C until required. At
137 the start of experimentation, the soil was acclimated under controlled laboratory
138 conditions (21 ± 1 °C, 45% humidity) for 8 d to allow for stabilisation of microbial
139 activity. Diesel oil was amended to soil (in batches of 500 g) by the procedure
140 previously described by Doick et al. (2003) to give nominal concentrations of 0, 0.05
141 and 0.5% (w/w). Batches of the amended soils containing the same amount of diesel
142 were pooled (~2.5 kg) and further blended together for 5 min to homogenise; two sets
143 of each of the soil amendments were produced. The amended soils were left exposed
144 for 24 h to simulate the initial weathering that might happen before spill containment
145 and/or remediation responses begin.
146 The next day, samples (2.5 kg) for unamended and the two amended soils were treated
147 with prescribed fire; the process of soil-burning was to simulate field scenarios where
148 the soil surfaces are exposed to prescribed or accidental fires after an oil spill. The
149 diesel-amended soil materials placed in a stainless tray to a depth not more than 10 mm
150 was burnt with propane flame held to the soil surfaces (Gray and Dighton, 2009). From
151 the preliminary trials of the procedure, it was estimated that the soil temperature
152 peaked at about 300 °C after 3–4 minutes and was sustained at 200–250 °C for up to 10
153 min; the temperature range that was maintained for this experiment can be described as

154 moderate (Robichaud and Hungerford, 2000) and within temperature ranges that have
155 been reported under prescribed fires (DeBano, 2000). After every 10 min of exposure,
156 the source of heat was withdrawn, the soil materials turned over severally and the
157 process of heating repeated two more times; this gave a total of 30 min exposure. Part
158 of the soil particles turned to black carbonaceous residues similar to those seen on the
159 surfaces of soil after wildfires (Fernandes et al., 2003). The burning exercise was
160 conducted outdoors (12–14 °C) and under the supervision of fire fighting officers. No
161 auxiliary fuel was used to initiate or sustain the combustion of the amended soil
162 materials.
163 To resuscitate injured microorganisms, the burnt soils were sprinkled with sterile
164 deionised water sufficient to bring the final moisture content of the soil to 60% of
165 water holding capacity (WHC); the moisture content of the unburnt soils was similarly
166 adjusted. The processes of diesel contamination and soil-burning produced different
167 soil treatments as listed in Table 1. The treated soils were placed in pre-cleaned amber
168 jars covered with perforated aluminium foil to allow for exchange of gases and then
169 acclimated in the dark at 21 ± 1 °C for 1, 30, 60 and 240 d. A wide-mouth flask
170 containing water to prevent excessive drying of soil was also placed in the box with the
171 amber jars. Periodically, the pH, moisture content and total hydrocarbon contents in the
172 treated soils were determined (data not reported).
173
174 *2.3. Microbiological analysis of soil treatments*
175 Colony forming units (CFUs) of naphthalene-, phenanthrene- and benzo[a]pyrene-
176 degrading bacteria were enumerated on agar plates impregnated with the respective
177 ^{12}C-PAHs (25 mg l^{-1}) as sole source or carbon following standard plate count

techniques (Lorch et al., 1995). The plates were incubated in the dark at 25 °C and enumeration of distinct colonies was carried out after 10–14 d.

2.4. Mineralisation of freshly added ^{14}C-PAHs in treated soils

The ability of the indigenous soil microflora to mineralise ^{14}C-PAHs to $^{14}CO_2$ was assessed after 1, 30, 60 and 240 d post-treatments. The ^{14}C-radiorespirometric assays were set-up in modified 250 ml Schott bottles containing soil (10 ± 0.2 g) and 30 ml sterile minimal basal salts (MBS) solution (Reid et al., 2001); the detailed composition of the MBS solution has been described elsewhere (Fenlon et al., 2011). PAH standards were prepared in toluene to deliver ^{12}C-naphthalene (50 mg kg^{-1}), ^{12}C-phenanthrene (50 mg kg^{-1}) or ^{12}C-benzo[a]pyrene (10 mg kg^{-1}); each ^{12}C-PAH standard contained an associated ^{14}C-activity of 83 kBq kg^{-1}. Respirometers were incubated at 21 ± 1 °C in the dark on a rotary shaker (100 rpm) and sample periodically over a period of 30 d. The $^{14}CO_2$ trapped in 1 ml NaOH (1 M) was mixed with 5 ml Goldstar scintillation cocktails and quantified by liquid scintillation counting (LSC, Tri-Carb 2300TR; Canberra Packard, Belgium).

2.5. Sequential extractions of ^{14}C-PAHs residues in soil slurry after mineralisation

The distribution of ^{14}C-PAH residues in all treated soils after the 30-d mineralisation assays was determined by a scheme of sequential extractions with solvents of decreasing polarity water (soluble fractions), methanol (MeOH) (polar fractions), dichloromethane (DCM) (nonpolar fractions), and by sample oxidation (solvent-nonextractable fractions), as described below. The choice of solvents and the sequence of extractions was based on the reducing polarity of the solvents (Northcott and Jones, 2001). The content in each respirometer was carefully transferred into a Teflon-lined

203 Oak Ridge centrifuge tube (50 ml) and 1 ml NaN_3 (0.01 M) added to inhibit further
204 microbial activity. After centrifugation at 3600 x *g* for 30 min, aliquot of the
205 supernatant collected was sampled (3 ml) into a 20-ml scintillation vial. Additional 30
206 ml of deionized water was used to thoroughly rinse the walls of the respirometer, and
207 the content emptied into the centrifuge tube; centrifugation repeated and an aliquot (3
208 ml) transferred into a 20-ml vial; the rest of the water was evaporated under the fume
209 hood to obtain solid soil pellet. The soil pellet was re-suspended in 30 ml of MeOH,
210 end-over-end shake-extracted for 24 h and centrifuged at 3600 x *g* for 30 min. The
211 process was repeated in 30 ml DCM (anhydrous Na_2SO_4 was also added). After each
212 centrifugation, an aliquot (3 ml) of the supernatant was withdrawn into a 20-ml vial
213 containing 15 ml Goldstar scintillation cocktails. The ^{14}C-PAH residues extracted with
214 the water, MeOH or DCM were quantified by LSC as previously described. The
215 influence of soil physicochemical properties on the partitioning of ^{14}C-PAHs was
216 assessed in metabolically inactive samples of the control soil earlier prepared by
217 autoclaving (121 °C for 15 min x 3 times on 3 consecutive days; sterility was
218 maintained with 0.5% NaN_3 (v/v) during the radiorespirometry.
219
220 *2.6. Sample oxidation of extracted soil pellets to quantify solvent-nonextractable ^{14}C-*
221 *PAH residues*
222 After drying the solvent-extracted soil pellet under the fume-hood, subsample (*ca.* 1 g)
223 was sample oxidised (Packard model 307, Berkshire, UK) to quantify the
224 nonextractable ^{14}C-PAH residues left after the series of sequential extractions. Prior to
225 combustion (3 min), the efficiency of the machine was determined (>97% at any time).
226 The ^{14}C-activity combusted to $^{14}CO_2$ was trapped in 10 ml Carbosorb-E® and 10 ml
227 Permafluor® was quantified by LSC, as previously described.

228

229 *2.7. Data presentation and analysis*

230 The ^{14}C-radiorespirometric data, initially corrected for background radioactivity and
231 machine noise, were used to calculate the overall extents (%), fastest rates (% d^{-1}) and
232 T_{max} (d; time taken to reach the fastest rate), as well as lag phase (d; time taken for
233 cumulative mineralisation to exceed 5% of added ^{14}C-PAH). The effects, individual
234 and combined, of the diesel concentrations, soil-burning and time post-treatments on
235 the mineralisation indices were evaluated using multivariate analysis of variance
236 (MANOVA) using the SigmaStat for Windows package (ver. 3.5, IBM SPSS Inc., US).
237 Holm-Sidak post-hoc comparisons test was used to distinguish significance differences
238 ($P < 0.05$). Normality of raw data was initially run and where normality test was not
239 established, data were transformed by either deriving their lognormal or reciprocal
240 values.

241

242 **3. Results**

243 After short-term (1-, 30- and 60-d) and long-term (240-d) post-treatments,
244 mineralisation of ^{14}C-naphthalene, ^{14}C-phenanthrene or ^{14}C-benzo[a]pyrene to $^{14}CO_2$
245 by the indigenous soil microorganisms was measured and the results presented
246 graphically in Figures 1–3. The results of bacterial cell counts (as CFUs g^{-1}) are shown
247 in Table 2 while the indices (lag phases, fastest rates and extents) of mineralisation are
248 presented in Table 3. The polarity of the ^{14}C-PAH residues recovered after
249 mineralisation (Table 4 and Figure 4) are also presented.

250

251 *3.1. Bacterial cell numbers in treated soils*

252 In general, the CFUs of naphthalene- and phenanthrene degrading bacteria were always
253 greater than those of benzo[a]pyrene-degrading bacteria, in the untreated (NUB) and all
254 treated soils (i.e. MUB, HUB, NBT, MBT and HBT) at all sampling times post-
255 treatment (Table 2). As compared to the NUB soil, the PAH-degrading bacterial
256 numbers increased and remained higher in the MUB and HUB soils, particularly for the
257 first 60-d post-treatments. At 1-d post-treatments, the CFUs of PAH-degrading bacteria
258 were lower in the NBT, MBT and HBT soils, but increased and were similar or greater
259 after 30-d and 60-d post-treatments, as compared to the NUB soil. After 240-d post-
260 treatments, the CFUs of PAH-degrading bacteria were generally comparable in the
261 NUB and all other treated soils.

262

263 *3.2. Mineralisation of ^{14}C-naphthalene in treated soils*

264 At every sampling time post-treatment, ^{14}C-naphthalene mineralisation was relatively
265 rapid (i.e. lag phase 1.65–2.89 d) and extensive (≥50%) in the NUB soil (Table 3,
266 Figure 1). In the MUB and HUB soils, the lag phases of ^{14}C-naphthalene mineralisation
267 decreased as time post-treatments increased; though for the HUB soil, the lag phase
268 was significantly longer ($P < 0.05$) compared to the NUB soil after 1-d post-treatments.
269 The decrease in the lag phases was particularly significant ($P < 0.05$) after 30-d post-
270 treatments for the MUB soil and after 60-d post-treatments for the HUB soil. Although,
271 the lag phase of ^{14}C-naphthalene mineralisation was significantly longer ($P < 0.05$)
272 after 1-d post-treatments, it became relatively shorter in the NBT compared to the NUB
273 soil, as time post-treatments increased. As compared to the NBT soil, the lag phase of
274 ^{14}C-naphthalene mineralisation in the MBT and HBT soils was longer after 1-d post-

275 treatments but progressively decreased and was significantly shorter ($P < 0.05$) as time
276 post-treatments increased.
277 The trend of the effects, individual and combined, of soil treatments on the fastest rate
278 of ^{14}C-naphthalene mineralisation was similar to those on the lag phase; though in most
279 cases the effects were not statistically significant ($P > 0.05$). Remarkably, the time for
280 the fastest rate to peak (T_{max}) presented a clearer trend of the effects of soil treatments
281 on ^{14}C-naphthalene mineralisation than the fastest rate as an index of mineralisation
282 (Table 3). In the NUB soil, the T_{max} value ranged 3–5 d for all sampling times post-
283 treatments; whereas the T_{max} was 6–7 d in the MUB soil and 18–25 d in the HUB soil
284 after 1 d post-treatments and significantly declined to 3 d in the MUB soil and 6–7 d in
285 the HUB soil after 30- and 60-d post-treatments and further to 1 d in both treated soils
286 after 240-d post-treatments. Likewise, the T_{max} was initially higher (4–6 d) but became
287 relatively shorter in the NBT (2–3 d), MBT (1–3 d) and HBT soils (1–3 d) than in the
288 NUB soil, as time post-treatments increased.
289 In the MUB and HUB soils there was no significant effect ($P > 0.05$) on the extents of
290 ^{14}C-naphthalene mineralisation at any of the sampling times post-treatments (Table 3,
291 Figure 1). In the NBT soil, ^{14}C-naphthalene mineralisation was significantly retarded
292 ($P < 0.05$) compared to the NUB soil, after 1-d post-treatments. Apparently, this effect
293 was transient since the extent of ^{14}C-naphthalene mineralisation was comparable ($P >$
294 0.05) in the NBT and NUB soils at the other times post-treatments. In the MBT and
295 HBT soils, the extent of ^{14}C-naphthalene mineralisation was dependent on the initial oil
296 concentration and time post-treatments. At 1-d post-treatments, the extent of ^{14}C-
297 naphthalene mineralisation was significantly lower ($P < 0.05$) in the MBT relative to
298 both NUB and HBT soils. After 30-d post-treatments, the extent of ^{14}C-naphthalene
299 mineralisation was significantly higher ($P < 0.05$) in both MBT and HBT soils

compared to NUB soil. Thereafter as time post-treatments increased, the extent of ^{14}C-naphthalene mineralisation became comparable ($P > 0.05$) in both MBT and HBT soils to NUB soil.

3.3. Mineralisation of ^{14}C-phenanthrene in treated soils

At every sampling time post-treatment, ^{14}C-phenanthrene mineralisation was extensive (≥50%) with relatively short lag phases (3.50–7.77 d) in the NUB soil (Table 3, Figure 2). In the MUB and HUB soils, the lag phase of ^{14}C-phenanthrene mineralisation was dependent on the initial oil concentration and time post-treatments. As compared to the NUB soil, the lag phase significantly decreased ($P < 0.05$) in the MUB soil as time post-treatments increased; however, the lag phase in the HUB soil was significantly longer ($P < 0.05$) after 1 d, significantly shorter ($P < 0.05$) at 30 d, and then significantly longer ($P < 0.05$) after 60-d and 240-d post-treatments. At 1-d post-treatments, the lag phase of ^{14}C-phenanthrene mineralisation was not different in the NBT soil but significantly shorter ($P < 0.05$) in the MBT and HBT soils as compared to the NUB soil. As time post-treatments increased, the lag phase of ^{14}C-phenanthrene mineralisation significantly decreased ($P < 0.05$) in the NBT soil as well as in both MBT and HBT soils compared to the NUB soil.

Diesel contamination with or without soil-burning had a similar effect on the fastest rate of ^{14}C-phenanthrene mineralisation as on the lag phase; though the wide variations in the rates in the replicate samples often resulted in statistically insignificant effects ($P > 0.05$). It is noteworthy that the T_{max} values were longer and varied more widely in the MUB and HUB soils than in the MBT and HBT soils after 1-d post-treatments; for example, the T_{max} value ranged 8–15 d in the HUB soil but was 5 d in the HBT soil (Table 3). Further, while the variation in the T_{max} generally lessened in both MUB and

HUB soils as time post-treatments increased, the variation gradually widened in the HBT soil.

As compared to the NUB soil, the extent of ^{14}C-phenanthrene mineralisation was not significantly different ($P > 0.05$) in the MUB soil at any of the sampling time post-treatments. In the HUB soil the extent of ^{14}C-phenanthrene mineralisation was significantly lower ($P < 0.05$) after 1-d post-treatments, then significant higher ($P < 0.05$) after 30-d post-treatments before it became comparable ($P > 0.05$) to the NUB soil, as time post-treatments increased. The extent of ^{14}C-phenanthrene mineralisation was comparable ($P > 0.05$) in the NUB and NBT soils, at any of the sampling time post-treatments. As compared to the NUB soil, the extent of ^{14}C-phenanthrene mineralisation was significantly higher ($P < 0.05$) in both MBT and HBT soils only after 1-d post-treatments.

3.4. Mineralisation of ^{14}C-benzo[a]pyrene in treated soils

In general, ^{14}C-benzo[a]pyrene mineralisation was inherently limited (extent <2% of the added ^{14}C-activty) in the NUB soil (Table 3, Figure 3). At any of the sampling times post-treatments, the lag phase of ^{14}C-benzo[a]pyrene mineralisation in both NUB and NBT soils were very long (>30 d). In the MUB and HUB soils, the lag phase of ^{14}C-benzo[a]pyrene mineralisation decreased as time post-treatments increased, being consistently significantly shorter ($P < 0.05$) after 1-d post-treatment for the MUB soil and after 30-d post-treatment for the HUB soil, as compared to NUB soil. Equally, there were significant decreases ($P < 0.05$) in the lag phase of ^{14}C-benzo[a]pyrene mineralisation in both MBT and HBT soils compared to the NUB soil at all sampling times post-treatments.

349 The fastest rate of ^{14}C-benzo[a]pyrene mineralisation appeared to be dependent on the
350 initial oil level amended to soil; though not statistically significant ($P > 0.05$). In the
351 MUB soil, the fastest rate of ^{14}C-benzo[a]pyrene was highest at 1-d post-treatments
352 while in the HUB soil it was achieved after 60-d post-treatments. As compared to the
353 NUB soil, the fastest rate of ^{14}C-benzo[a]pyrene in the NBT soil was usually higher
354 though not significantly ($P > 0.05$), at all times post-treatments. Also, the fastest rate of
355 ^{14}C-benzo[a]pyrene was usually higher in both MBT and HBT soils than in the NUB
356 and NBT soils. Compared to the fastest rate, the T_{max} parameter gave a better
357 interpretation of the effects of the soil treatments on ^{14}C-benzo[a]pyrene mineralisation
358 in this study (Table 3). The range of the T_{max} values (from triplicate samples) for a
359 particular soil treatment showed variability in the potential of indigenous
360 microorganisms to mineralise ^{14}C-benzo[a]pyrene. In general, it appeared that the T_{max}
361 reduced and the range became narrower as time post-treatments increased.
362 The extent of ^{14}C-benzo[a]pyrene mineralisation was dependent on the initial oil level
363 and time post-treatments (Table 3, Figure 3). The extent of ^{14}C-benzo[a]pyrene
364 mineralisation was negligible (<2%) in the NUB soil, at any of the sampling time post-
365 treatments. At all times post-treatments, the extent of ^{14}C-benzo[a]pyrene
366 mineralisation was significantly higher ($P < 0.05$) in the MUB, HUB, MBT and HBT
367 soils than in the NUB and NBT soils. Although usually statistically not significant ($P >$
368 0.05), the extent of ^{14}C-benzo[a]pyrene mineralisation was higher in the NBT
369 compared to the NUB soil. In the short-term (1-, 30- and 60-d post-treatments), the
370 extents of ^{14}C-benzo[a]pyrene mineralisation was significantly different ($P < 0.05$) in
371 the MUB and the HUB soils; the extent decreased in the MUB soil, but increased in the
372 HUB soil, as time post-treatments increased. In the long-term (240-d post-treatments),
373 the extent of ^{14}C-benzo[a]pyrene mineralisation was no longer significantly different (P

374 > 0.05) in both MUB and HUB soils. The extent of ^{14}C-benzo[a]pyrene mineralisation
375 was significantly different ($P < 0.05$) in the MBT and HBT soils, at any of the
376 sampling time post-treatments; this being relatively lower in the MBT soil (3–15%)
377 than in the HBT soil (15–36%).
378
379 *3.5. Polarity of ^{14}C-PAH residues in soil slurry after mineralisation*
380 The ^{14}C-balances of PAHs in the metabolically inactive (i.e. autoclaved + NaN_3–
381 treated) soil include data of abiotic loss, aqueous and organic solvent extractions of soil
382 slurry followed by sample oxidation of remaining soil pellets (Table 4). In the sterilised
383 soil, 90.72 ± 1.51%, 100.37 ± 1.57% and 98.56 ± 2.34% of the added ^{14}C-naphthalene,
384 phenanthrene and ^{14}C-benzo[a]pyrene, respectively, were recovered after 30 d
385 incubation. There was no abiotic loss of ^{14}C-phenanthrene or ^{14}C-benzo[a]pyrene but
386 there was a negligible volatilisation of ^{14}C-naphthalene (3%). The aqueous distribution
387 of the ^{14}C-PAHs reflected their aqueous solubility, and the ratio of in the aqueous-
388 phase fraction to the organic solvents-extractable fraction correlated with the octanol–
389 water partitioning coefficients of the PAHs. All of the added ^{14}C-naphthalene or ^{14}C-
390 phenanthrene was extractable in the sterilised soil but a minute fraction of ^{14}C-
391 benzo[a]pyrene (3%) remained solvent-nonextractable in soil.
392 After mineralisation was terminated for all soil treatments, the amounts of ^{14}C-PAH
393 residues that partitioned into the aqueous phase, that were extractable by MeOH and
394 DCM, and that were solvent-nonextractable are presented in Figure 4 (results are
395 presented as the normalised values in stacked bars while the actual values are also
396 reported on top of the bars). Collectively for all the treated soils and at all sampling
397 times post-treatments, the portions of the added ^{14}C-PAHs recovered ranged from
398 80.05 to 101.03%, from 78.39 to 110.49%, and from 86.65 to 109.52% for ^{14}C-

399 naphthalene, ^{14}C-phenanthrene, and ^{14}C-benzo[a]pyrene, respectively; this indicated
400 good recovery particularly for ^{14}C-naphthalene which is volatile. Overall,
401 approximately 15–45% of the added ^{14}C-naphathalene, 20–60% of the added ^{14}C-
402 phenanthrene and 38–110% of the added ^{14}C-benzo[a]pyrene were recovered as ^{14}C-
403 residues after mineralisation.
404 The amounts recovered as ^{14}C-residues and their distributions to aqueous, MeOH and
405 DCM, as well as the solvent-nonextractable fractions were neither influenced by soil
406 treatments nor time post-treatments ($P > 0.05$). The main difference in the distribution
407 of ^{14}C-residues related to the chemical structure of parent ^{14}C-PAHs. For example,
408 greater amounts of ^{14}C-naphthalene residues (6–28%) and ^{14}C-phenathrene residues
409 (5–19%) than ^{14}C-benzo[a]pyrene residues (<1–5%) remained in the aqueous phase.
410 Similarly, greater portions of ^{14}C-naphthalene residues (31–78%) and ^{14}C-
411 phenanthrene (35–80%) than ^{14}C-benzo[a]pyrene residues (5–24%) were solvent-
412 nonextractable. On the other hand, larger amounts of ^{14}C-benzo[a]pyrene residues (45–
413 90%) than ^{14}C-phenanthrene (10–70%) and ^{14}C-naphthalene residues (10–70%) were
414 extractable by MeOH (as polar ^{14}C-metabolites) and DCM (as nonpolar ^{14}C-
415 metabolites). In general, it appeared there was less polar ^{14}C-metabolites than nonpolar
416 ^{14}C-metabolites; for example, of the ^{14}C-naphthalene residues recovered after
417 mineralisation, 2–20% was polar, 7–50% nonpolar while 16–45% of ^{14}C-
418 benzo[a]pyrene residues were polar and 31–60% were nonpolar.
419
420 **4. Discussion**
421 The ^{14}C-radiorespirometic assays similar to the one used in this present study have
422 been widely used to measure the impact on or the development of indigenous
423 degradative ability for PAHs and other hydrophobic organic contaminants in soil and

424 sediments (Grosser et al., 1991; Reid et al., 2001; Stroud et al., 2009; Posada-Baquero
425 and Ortega-Calvo, 2011; Reid et al., 2013). The rapid and extensive mineralisation of
426 ^{14}C-naphthalene or ^{14}C-phenanthrene but not ^{14}C-benzo[a]pyrene, together with the
427 abundance and relative proportion of naphthalene- or phenanthrene- to benzo[a]pyrene-
428 degrading bacterial populations, indicate that the control soil harboured bacteria
429 possessing high degradative ability to mineralise the LMW-PAHs, but not the HMW-
430 PAH; this is in agreement with the findings of other investigators who have worked on
431 this soil (Stroud et al., 2007; Couling et al., 2010; Towell et al., 2011). The absence of
432 substantial mineralisation of benzo[a]pyrene in the control soil may be attributed to the
433 relatively low abundance or poor degradative ability and/or inadequate assemblages of
434 catabolically-competent microorganisms (Kanaly and Harayama, 2000). In addition,
435 the inability of benzo[a]pyrene to readily support microbial growth, the requirement for
436 certain co-substrates for effective degradation, and the unfavourable physicochemical
437 properties can limit benzo[a]pyrene mineralisation in soil (Bamforth and Singleton,
438 2005).
439 At any of the sampling times post-treatments, the ^{14}C-radiorespirometric data revealed
440 a sequential pattern of PAH catabolism in all the treated soils, that is, a significant
441 fraction of ^{14}C-naphthalene was recovered as $^{14}CO_2$ before the onset in mineralisation
442 of ^{14}C-phenanthrene which also advanced to a maximum before the onset of ^{14}C-
443 benzo[a]pyrene mineralisation. This pattern has previously been observed for
444 phenanthrene and pyrene in a creosote contaminated soil (Bueno-Montes et al., 2011)
445 and during bioremediation of unweathered, PAH-polluted soils (Uyttebroek et al.,
446 2007). This phenomenon is attributable to competitive inhibition in which the initial
447 faster biodegradation of the LMW-PAHs may inhibit the degradation of the HMW-
448 PAHs (Leahy and Colwell, 1990; Stringfellow and Aitken, 1995; Kanaly et al., 2000).

449 Collectively, the data presented in this present study indicated that diesel amendment
450 and soil-burning, individually and jointly, can modulate the abundance of indigenous
451 microorganisms and the development of their degradative ability towards various
452 PAHs, especially in the short-term. In the long-term, the high degradative ability for
453 the low molecular weight (LMW)-PAHs (i.e. naphthalene and phenanthrene) in this
454 soil was only marginally affected, whereas the inherently low degradative ability the
455 high molecular weight (HMW)-PAH (i.e. benzo[a]pyrene) was significantly enhanced
456 by diesel amendment and/or soil-burning. Further, diesel amendment and soil-burning,
457 individually and jointly, have no apparent effects on ^{14}C-PAH residues polarity and
458 distribution in the soil. The marginal effect of diesel, at the concentrations investigated,
459 on the extents of ^{14}C-naphthalene and ^{14}C-phenanthrene mineralisation was most likely
460 due to the inherently high degradative ability for the LMW-PAHs in the soil.
461 Consistent with the results of this study, enhanced benzo[a]pyrene mineralisation has
462 been attributed to diesel acting as co-solvent for PAH dissolution and/or as inducers of
463 co-metabolism as well as promoting the growth of catabolically-competent microbial
464 populations in soil (Kanaly and Watanabe, 2004).
465 The relatively low impact on the PAH degradative ability of indigenous soil
466 microorganisms may be as a result of the low-severity of the prescribed fire applied to
467 the soil. Although there is evidence that prescribed fires can cause immediate effects on
468 soil microbial communities, both as a direct result of heating and indirectly via changes
469 to soil physical and chemical properties, as well as nutrient availability, the capacity for
470 recovery (i.e. resilience) is largely dependent on the degree of severity (intensity and
471 duration of exposure) (Bååth et al., 1995; Boerner et al., 2000; Pietikäinen et al., 2000;
472 Lindau et al., 2003; Zengel et al., 2003; Certini, 2005; Campbell et al., 2008; Gray and
473 Dighton, 2009; Swallow et al., 2009; Baustian et al., 2010; Rietl and Jackson, 2012;

474 Chalbot et al., 2013). Mostly, prescribed fires with low- to moderate-severity do not
475 result in irreversible ecosystem change though there may be transient increase in pH
476 and available nutrients (Certini, 2005; Baustian et al., 2010). While prescribed fire may
477 have negative effect on air quality and the atmospheric abundance of PAHs, as well as
478 on the soil properties (Certini, 2005; Whicker et al., 2006), in practical terms under
479 certain site conditions, prescribed fires may be either the best practicable
480 environmental option (BPEO) or the best alternative technology not entailing excessive
481 cost (BATNEEC). Meanwhile, a number of factors, which may vary widely, and are
482 largely dependent on specific sites, must be considered before a decision is taken to use
483 prescribed fire. Environmental factors like, climate, vegetation, and topography of the
484 burnt area, as well as soil characteristics, such as soil depth, moisture content and the
485 flammability of the spilled oil and other organic materials present may influence the
486 impacts of prescribed fires on many physical, chemical, mineralogical and biological
487 soil properties (Zengel et al., 2003; Certini, 2005).
488 In the context of remediation to achieve the minimal level of residual contaminants in
489 soil, collectively, the results of the study suggest that prescribed fire is an alternative
490 first-line remediation operation to consider, particularly for heavily-contaminated soil
491 environments. This is a preliminary study; hence further research is required to
492 determine the effects of variations in fire severity, moisture content as well as soil
493 types. Investigation of field-contaminated sites with vegetation cover or not, and with
494 variable histories of repeated fire events will provide further information on the impact
495 of prescribed fire on the development of indigenous PAH degradative ability.
496

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630

631 **Tables**

632 Table 1: Description of soil treatments.

Soil treatment	Soil treatment description	Soil tag
None	No diesel; No soil-burning	NUB
Diesel contamination only	500 mg kg^{-1} diesel; No soil-burning	MUB
	5000 mg kg^{-1} diesel; No soil-burning	HUB
Soil-burning only	No diesel; Soil-burning (200–250°C)	NBT
Diesel contamination and soil-burning	500 mg kg^{-1} diesel; Soil-burning (200–250°C)	MBT
	5000 mg kg^{-1} diesel; Soil-burning (200–250°C)	HBT

633

634 Table 2: Numbers (CFU g^{-1}) of PAH-degrading bacteria in the treated soils; values are

635 the means ±SEM (n = 4).

Time (d)	Soil tag	Naphthalene (x 10^6)	Phenanthrene (x 10^6)	Benzo[a]pyrene (x 10^3)
1	NUB	4.10 ± 1.50	4.60 ± 1.40	1.20 ± 0.10
	MUB	10.50 ± 3.70	9.50 ± 2.60	2.54 ± 1.40
	HUB	8.10 ± 1.00	8.70 ± 3.30	2.35 ± 0.90
	NBT	0.01 ± 0.01	0.08 ± 0.07	0.11 ± 0.05
	MBT	0.05 ± 0.03	0.10 ± 0.06	0.18 ± 0.10
	HBT	0.14 ± 0.12	0.08 ± 0.06	0.23 ± 0.11
30	NUB	3.45 ± 0.91	5.20 ± 2.00	3.00 ± 1.33
	MUB	15.00 ± 3.14	13.50 ± 2.45	8.30 ± 0.11
	HUB	15.20 ± 2.50	24.30 ± 4.40	10.20 ± 0.11
	NBT	2.12 ± 1.00	7.10 ± 2.22	5.12 ± 1.31
	MBT	6.30 ± 1.05	18.40 ± 3.87	3.51 ± 1.10
	HBT	13.21 ± 4.10	22.70 ± 6.13	5.45 ± 2.16
60	NUB	2.79 ± 0.50	4.18 ± 1.00	1.23 ± 0.43
	MUB	18.40 ± 5.40	19.08 ± 1.14	4.10 ± 1.34
	HUB	20.00 ± 4.60	26.90 ± 5.70	11.89 ± 3.33
	NBT	4.10 ± 2.18	8.05 ± 3.21	2.05 ± 0.88
	MBT	6.70 ± 1.34	14.80 ± 3.20	7.01 ± 3.10
	HBT	12.33 ± 4.00	15.32 ± 4.12	8.89 ± 3.11
240	NUB	5.25 ± 3.02	7.30 ± 1.34	1.60 ± 0.78
	MUB	8.10 ± 3.10	9.38 ± 3.22	4.76 ± 1.10
	HUB	7.90 ± 1.12	11.10 ± 4.71	8.14 ± 1.46
	NBT	ND	ND	ND
	MBT	5.82 ± 1.34	7.00 ± 2.11	3.11 ± 1.11
	HBT	6.64 ± 2.12	5.80 ± 1.34	9.65 ± 3.66

636

637 Table 3: Effects of the soil treatments on ^{14}C-PAH mineralisation after 1, 30, 60 and 240 d post-treatments.

Time (d)	Soil tag	^{14}C-Naphthalene			^{14}C-Phenanthrene			^{14}C-Benzo[a]pyrene		
		Overall extent, $\sum^{14}CO_2$ (%)	Fastest rate, (% d^{-1}) ; T_{max} (d)	Lag phase, (d)	Overall extent, $\sum^{14}CO_2$ (%)	Fastest rate, (% d^{-1}) ; T_{max} (d)	Lag phase, (d)	Overall extent, $\sum^{14}CO_2$ (%)	Fastest rate, (% d^{-1}) ; T_{max} (d)	Lag phase, (d)
1	NUB	73.56 ± 4.69 ^{aA‡}	24.38 ± 7.71 ^{aAB} (3 – 4)†	1.65 ± 0.17 ^{aA}	60.98 ± 0.53 ^{aA‡}	14.63 ± 3.40 ^{aAB} (8–9)†	5.88 ± 0.11 ^{aA}	0.43 ± 0.18 ^{aA‡}	0.03 ± 0.02 ^{aA} (*2–8*)†	>30 ^{aA}
	MUB	71.91 ± 1.64 ^{aA}	15.06 ± 9.06 ^{aA} (6–7)	2.51 ± 0.23 ^{aA}	61.46 ± 0.69 ^{aA}	9.19 ± 3.46 ^{aA} (12–14)	8.61 ± 0.57 ^{aA}	59.24 ± 3.92 ^{bA}	7.84 ± 4.15 ^{aA} (14–18)	13.72 ± 1.17 ^{bA}
	HUB	68.28 ± 3.83 ^{aA}	7.29 ± 3.12 ^{aA} (18 – 25)	6.40 ± 0.91 ^{bA}	38.93 ± 6.89 ^{bA}	4.87 ± 1.63 ^{aA} (8–15)	23.32 ± 1.67 ^{bA}	0.87 ± 0.29 ^{aA}	0.07 ± 0.05 ^{aA} (*21–30*)	>30 ^{aA}
	NBT	45.62 ± 1.16 ^{cA***}	5.84 ± 0.54 ^{aA} (5–6)	4.05 ± 0.26 ^{aA}	59.21 ± 0.98 ^{aA}	9.09 ± 2.83 ^{aA} (12)	8.29 ± 1.42 ^{aA}	2.98 ± 0.26 ^{aA}	0.15 ± 0.05 ^{aA} (*18–21*)	>30 ^{aA}
	MBT	56.90 ± 0.87 ^{bA***}	12.37 ± 5.98 ^{aA} (4–5)	3.38 ± 0.05 ^{bA}	72.97 ± 0.65 ^{bA***}	32.00 ± 2.06 ^{aA} (6)	4.58 ± 0.20 ^{bA}	5.13 ± 1.62 ^{aA***}	0.11 ± 0.09 ^{aA} (25)	>23.98 ^{bA}
	HBT	71.39 ± 3.77 ^{aA}	13.90 ± 4.70 ^{aA} (4–5)	3.24 ± 0.04 ^{bA}	72.85 ± 2.72 ^{bA**}	18.61 ± 8.54 ^{aA} (5)	3.97 ± 0.41 ^{bA}	18.69 ± 4.23 ^{bA**}	1.45 ± 0.41 ^{bA} (15)	14.80 ± 1.26 ^{cA}
30	NUB	49.98 ± 2.52 ^{aC}	11.72 ± 6.45 ^{aB} (4–5)	2.89 ± 0.16 ^{aC}	49.65 ± 4.05 ^{aB}	7.98 ± 1.52 ^{aB} (8–9)	7.77 ± 0.25 ^{aB}	1.15 ± 0.31 ^{aA}	0.21 ± 0.14 ^{aA} (*7–8*)	>30 ^{aA}
	MUB	52.51 ± 4.29 ^{aB}	17.80 ± 2.88 ^{aA} (3)	0.99 ± 0.06 ^{bB}	55.54 ± 1.86 ^{abA}	11.11 ± 1.12 ^{bA} (8)	4.32 ± 0.16 ^{bB}	8.56 ± 3.89 ^{aB}	0.46 ± 0.25 ^{aA} (6–25)	19.20 ± 4.56 ^{bA}
	HUB	55.78 ± 1.26 ^{aB}	13.03 ± 4.26 ^{aA} (4–5)	2.79 ± 0.01 ^{aB}	65.05 ± 1.47 ^{bB}	36.63 ± 1.20 ^{bB} (4)	2.99 ± 0.11 ^{cB}	26.24 ± 5.64 ^{bB}	1.48 ± 1.00 ^{aA} (6–30)	10.53 ± 2.07 ^{cB}
	NBT	57.95 ± 3.52 ^{aA}	20.81 ± 2.78 ^{aB} (3)	1.75 ± 0.07 ^{aB}	45.68 ± 0.46 ^{aB}	13.19 ± 0.74 ^{aA} (4)	2.64 ± 0.06 ^{aB}	1.87 ± 0.43 ^{aA}	0.21 ± 0.06 ^{aA} (*10*)	>30 ^{aA}
	MBT	67.28 ± 2.18 ^{aBC*}	18.06 ± 3.82 ^{aA} (3)	1.34 ± 0.02 ^{bB}	52.30 ± 2.80 ^{aB}	19.35 ± 1.67 ^{aB} (4)	2.96 ± 0.09 ^{bB}	3.00 ± 0.96 ^{aA}	0.35 ± 0.15 ^{aA} (*7–10*)	>30 ^{aB}
	HBT	67.49 ± 1.47 ^{aA**}	26.51 ± 2.72 ^{aAB} (3)	1.80 ± 0.11 ^{aB}	53.21 ± 1.91 ^{aB**}	12.36 ± 1.96 ^{aA} (4–5)	3.33 ± 0.09 ^{cA}	25.45 ± 2.60 ^{bAB}	2.42 ± 0.25 ^{bA} (10)	9.45 ± 0.25 ^{cA}
60	NUB	57.72 ± 4.02 ^{aBC}	16.42 ± 1.60 ^{aB} (4)	2.18 ± 0.05 ^{aB}	62.29 ± 3.45 ^{abA}	15.43 ± 2.61 ^{aAB} (6–7)	5.23 ± 0.20 ^{abC}	0.79 ± 0.16 ^{aA}	0.05 ± 0.01 ^{aA} (*7–8*)	>30 ^{aA}
	MUB	63.59 ± 3.77 ^{aAB}	19.34 ± 2.34 ^{aA} (3)	0.89 ± 0.11 ^{cB}	64.24 ± 3.74 ^{abA}	10.48 ± 4.67 ^{aA} (6–7)	3.99 ± 0.28 ^{aB}	8.13 ± 5.47 ^{aB}	0.46 ± 0.41 ^{aA} (8–26)	>17.58 ^{bA}
	HUB	64.30 ± 3.06 ^{aAB}	10.83 ± 4.18 ^{aA} (4–5)	1.32 ± 0.12 ^{cBC}	54.57 ± 2.61 ^{aAB}	5.28 ± 2.91 ^{aA} (11–12)	8.93 ± 1.64 ^{bC}	52.29 ± 3.46 ^{bC}	3.90 ± 1.85 ^{aA} (7–15)	8.02 ± 1.86 ^{cB}
	NBT	48.51 ± 6.39 ^{aA}	12.99 ± 2.18 ^{aB} (2)	1.31 ± 0.05 ^{aB}	55.64 ± 3.58 ^{aA}	16.35 ± 4.18 ^{aA} (3–4)	2.05 ± 0.01 ^{aB}	3.53 ± 1.71 ^{aA}	0.22 ± 0.09 ^{aA} (*8–22*)	>30 ^{aA}
	MBT	62.70 ± 0.26 ^{aAB}	21.30 ± 0.57 ^{bA} (2)	1.15 ± 0.01 ^{bC}	71.26 ± 4.22 ^{bA}	21.69 ± 1.30 ^{aB} (3)	1.36 ± 0.05 ^{bC}	2.98 ± 0.94 ^{aA}	0.15 ± 0.05 ^{aA} (*8–18*)	>30 ^{aB}
	HBT	61.86 ± 6.41 ^{aA}	20.77 ± 4.48 ^{aA} (3)	1.42 ± 0.01 ^{cC}	72.48 ± 2.73 ^{bA***}	15.45 ± 2.36 ^{aA} (3–4)	2.15 ± 0.04 ^{aB}	14.92 ± 1.26 ^{bA***}	0.81 ± 0.09 ^{bA} (11–22)	14.62 ± 1.10 ^{bA}
240	NUB	65.41 ± 0.29 ^{aAB}	41.83 ± 2.44 ^{aA} (3)	2.04 ± 0.02 ^{aAB}	69.29 ± 2.46 ^{aA}	25.12 ± 3.68 ^{aA} (5)	3.50 ± 0.16 ^{aD}	1.52 ± 0.47 ^{aA}	0.09 ± 0.03 ^{aA} (*5–9*)	>30 ^{aA}
	MUB	65.29 ± 2.93 ^{aAB}	18.74 ± 2.58 ^{bA} (1)	0.28 ± 0.04 ^{bC}	64.74 ± 0.94 ^{aA}	23.38 ± 1.79 ^{aA} (2)	1.12 ± 0.03 ^{bC}	24.09 ± 3.23 ^{bB}	2.03 ± 0.47 ^{bA} (6–9)	7.64 ± 0.87 ^{cA}
	HUB	70.90 ± 0.24 ^{aA}	41.31 ± 4.18 ^{aB} (1)	0.12 ± 0.01 ^{cC}	49.98 ± 7.22 ^{aAB}	15.99 ± 5.20 ^{aA} (10–11)	9.18 ± 0.10 ^{cC}	17.18 ± 5.58 ^{bAB}	1.62 ± 0.06 ^{bA} (7)	12.74 ± 2.09 ^{bB}
	NBT	ND	ND	ND	ND	ND	ND	ND	ND	ND
	MBT	72.14 ± 1.90 ^{aC}	22.67 ± 4.34 ^{aA} (1)	0.24 ± 0.05 ^{aD}	64.96 ± 2.30 ^{aA}	32.48 ± 3.23 ^{aA} (2)	1.07 ± 0.01 ^{aC}	14.91 ± 2.77 ^{aB}	0.52 ± 0.38 ^{aA} (12–30)	15.97 ± 2.68 ^{aC}
	HBT	74.10 ± 3.59 ^{aA}	41.66 ± 2.86 ^{bB} (1)	0.12 ± 0.01 ^{aD}	52.43 ± 6.33 ^{aB}	18.18 ± 2.44 ^{bA} (7–8)	5.89 ± 0.10 ^{bC}	36.24 ± 4.46 ^{bB}	2.33 ± 2.16 ^{aA} (5–30)	15.71 ± 3.90 ^{aA}

638 ‡ Different lower-case letters down the column within each sub-group of "ageing period" indicate the means of triplicate samples that are significantly different ($P < 0.05$); different upper-case letters down the
639 column for corresponding "concentration" across groups of "ageing periods" indicate the means of triplicate samples that are significantly different ($P < 0.05$).
640 † Values in parentheses are time taken for mineralisation to reach fastest rates in days ($n = 3$).
641 ND: not determined.

642

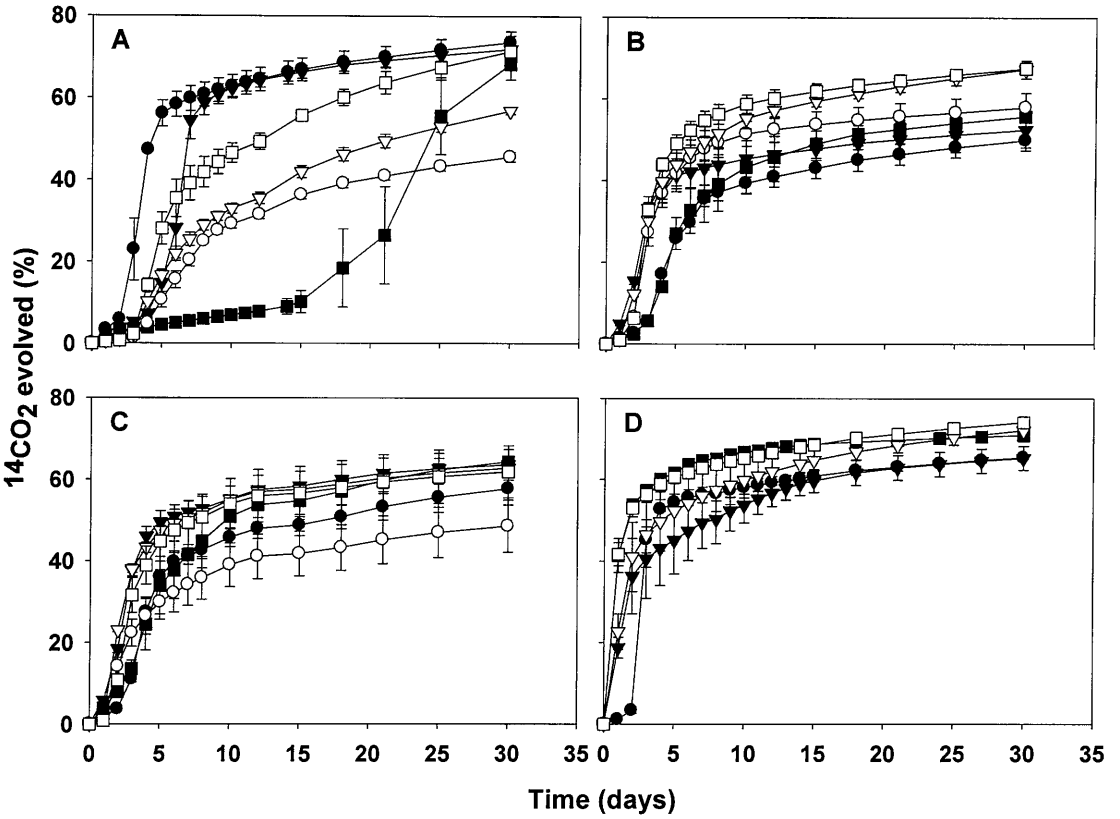
643 Table 4: Distribution of ^{14}C-PAHs in metabolically inactive (autoclaving + NaN_3–treated) soil.

PAH	$^{14}CO_2$	Aqueous	MeOH	DCM	Nonextractable	Total
Naphthalene	2.96 ± 0.27	21.41 ± 1.61	48.81 ± 5.59	16.63 ± 2.41	0.99 ± 0.76	90.72 ± 1.51
Phenanthrene	0.39 ± 0.04	4.06 ± 0.28	76.06 ± 1.74	18.90 ± 0.25	0.94 ± 0.13	100.37 ± 1.57
Benzo[a]pyrene	0.03 ± 0.01	0.75 ± 1.42	32.39 ± 1.99	62.48 ± 0.88	2.94 ± 0.38	98.58 ± 2.34

644

645 **Figures**

646



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648

649 Figure 1: Development of ^{14}C-naphthalene catabolism in soil after (A) 1, (B) 30, (C) 60

650 and (D) 240 d post-treatments. Legends: NUB (●), MUB (▼), HUB (■), NBT (○),

651 MBT (▽) and HBT (□) soil treatments.

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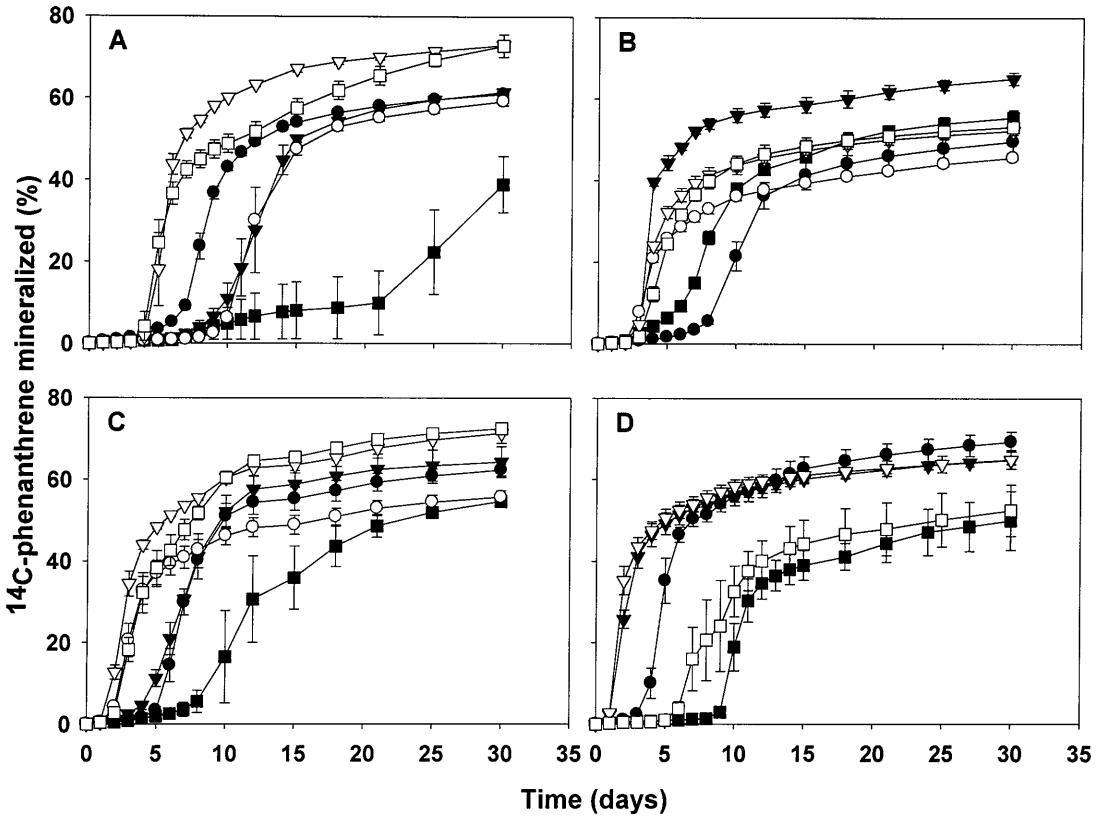

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655

656 Figure 2: Development of ^{14}C-phenanthrene catabolism in soil after (A) 1, (B) 30, (C)

657 60 and (D) 240 d post-treatments. Legends: NUB (●), MUB (▼), HUB (■), NBT (○),

658 MBT (▽) and HBT (□) soil treatments.

659

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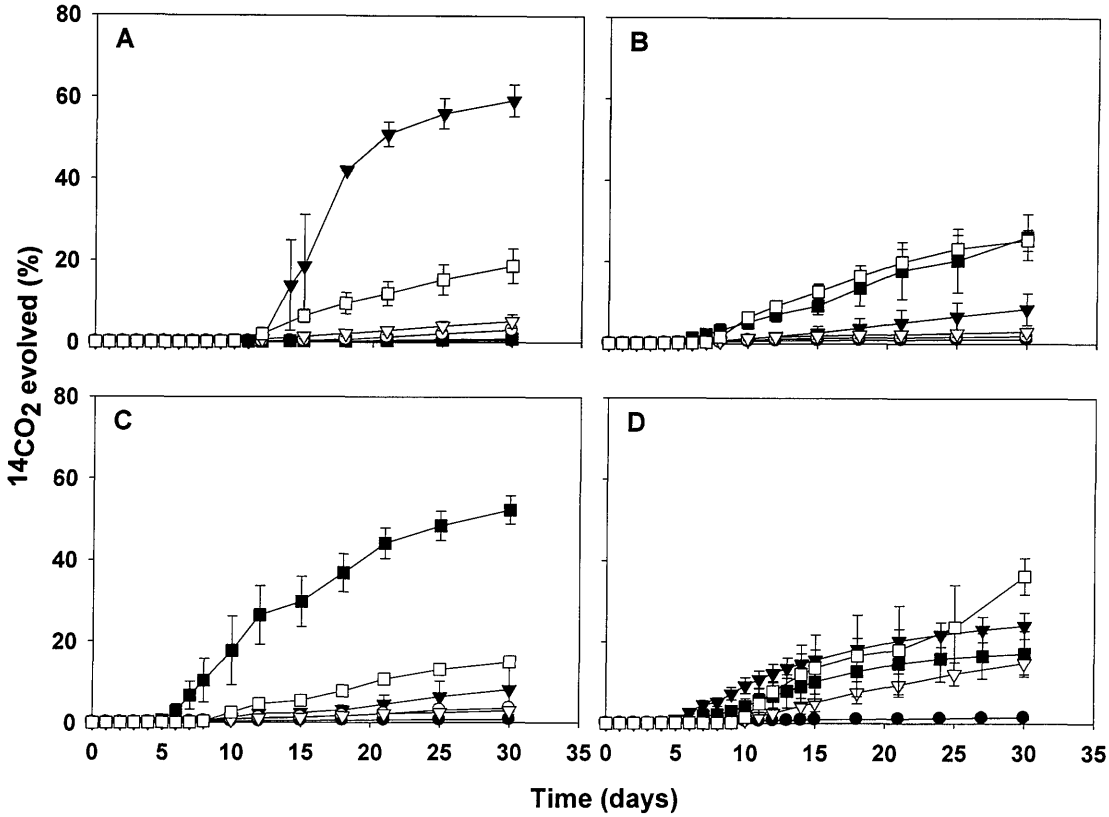
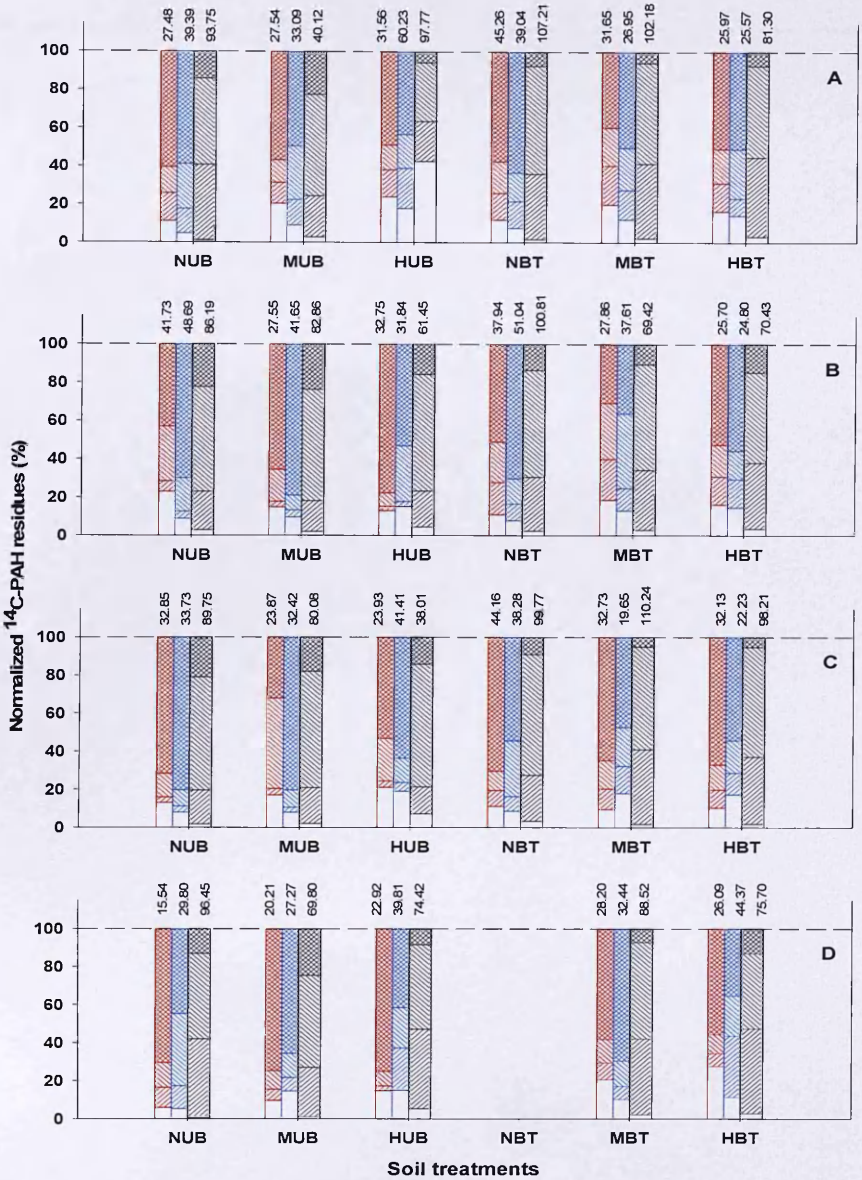


661
662

663 Figure 3: Development of ^{14}C-benzo[a]pyrene catabolism in soil after (A) 1, (B) 30,

664 (C) 60 and (D) 240 d post-treatments. Legends: NUB (●), MUB (▼), HUB (■), NBT

665 (○), MBT (▽) and HBT (□) soil treatments.



667

668 Figure 4: Distribution of ^{14}C-PAH residues in soil slurry after 30-d mineralisation
669 assay following (A) 1-d (B) 30-d (C) 60-d and (D) 240-d post-treatments. Legends:
670 Aqueous (white), MeOH (bottom-left-to-top-right hatched), DCM (top-left-to-bottom-
671 right hatched) and solvent-nonextractable (cross-hatched) ^{14}C-residues. Each set of
672 grouped bars represents ^{14}C-naphthalene (left or red bars), ^{14}C-phenanthrene (middle or
673 blue bars), and ^{14}C-benzo[a]pyrene residues (right or black bars), respectively. Value
674 on top of each stacked bar indicates the percent of the added ^{14}C-PAH recovered as
675 ^{14}C-residues after mineralisation.

676

Paper X

Effect of diesel oil concentration on the quantification of ^{14}C-biomass in soil

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12 **Abstract**

13 Accurate quantification of soil microbial biomass carbon is essential in assessing
14 nutrient fate and transformations, predicting energy flux and understanding ecosystem
15 processes and functioning in soil. The influence of diesel oil concentration on ^{14}C-
16 biomass quantification using the chloroform fumigation-extraction (FE) technique was
17 evaluated in four soils with differing physicochemical and microbiological
18 characteristics. ^{14}C-Biomass was determined after 5, 15 and 30 d of incubation in soils
19 amended with diesel at 10, 100, 1000 and 5000 mg kg^{-1} and spiked with ^{14}C-glucose.
20 The presence of high diesel concentrations (1000–5000 mg kg^{-1}) interfered
21 significantly ($P < 0.05$) with the extraction efficiency (k_{EC}) for ^{14}C-biomass
22 quantification by FE technique. Based on the experimental data, the use of *in-situ*
23 derived k_{EC} values rather than a fixed k_{EC} value for ^{14}C-biomass quantification in diesel
24 oil-contaminated soils is advised.

25

26 **Capsule:**

27 The use of *in-situ* derived k_{EC} rather than a fixed k_{EC} for quantification of soil microbial
28 biomass-carbon in diesel oil contaminated soils is advised.

29

30 **Keywords**: *Chloroform fumigation extraction; Extraction efficiency (k_{EC}); Diesel oil;*
31 *^{14}C-glucose; Microbial biomass; Soil*

32

Highlights

- *Effect of diesel concentration on ^{14}C-biomass quantification by FE is evaluated.*
- *High diesel concentrations (≥1000 mg kg^{-1}) interfere with FE extraction efficiency.*
- *Extent of interference depends on incubation time and soil type.*
- *Use of derived* k_{EC} to *quantify ^{14}C-biomass in diesel-contaminated soil is advised.*

1. Introduction

The soil organic carbon (SOC) plays a fundamental role in the dynamics of global carbon biogeochemical cycling, in climate change, land use and soil quality management (Cerri et al., 2007a; Cerri et al., 2007b). Soil microbial biomass-carbon (biomass-C) is the primary source of SOC (Franzluebbers et al., 2001; Joergensen et al., 2011). To date, there are no direct means of measuring accurately biomass-C. However, accurate quantification of biomass-C is essential in assessing nutrient fate and transformations (Alessi et al., 2011) or in predicting flux of energy in soil and in understanding ecosystem processes and functioning (Müller et al., 2006; Cerri et al., 2007a). It is also useful as internal control to validate data derivable from other microbial activity parameters (Joergensen et al., 2011).

The chloroform fumigation-extraction (FE) technique, originally developed by Vance et al. (1987), has been widely used for the biomass-C quantification in uncontaminated soils (Mariani et al., 2006; Mamilov and Dilly, 2007; Joergensen, 2010; Sullivan and Hart, 2013), and to lesser extents, in contaminated soils (Bardgett and Saggar, 1994; Joergensen et al., 1995; Boucard et al., 2008; Pratt et al., 2012; Thiessen et al., 2013). The FE technique is based on the ability of ethanol-free chloroform to lyse microbial cells and release their C contents during 24-h soil fumigation. An extraction efficiency factor (k_{EC}) is then used to convert the extra C released (C-flush) into a simple salt solution (e.g. 0.5 M K_2SO_4) to biomass-C. Several values of k_{EC} have been proposed depending on whether the calibration is direct or indirect (Joergensen et al., 2011) and ranged from 0.15 to 0.98 (see Sparling et al., 1981b; Sparling et al., 1981a; West et al., 1986; Sparling and West, 1988; Tate et al., 1988; Gregorich et al., 1990; Sparling et al., 1990; Bremer and Kuikman, 1994; Joergensen, 1996) but the value of 0.45 (Wu et al., 1990) has been widely employed in studies on uncontaminated and contaminated soils

65 (Joergensen et al., 2011) without the careful evaluation of the influences that soil
66 characteristics and contaminants may have on the extraction process.
67 One of the major challenges with the FE technique is the choice of the k_{EC} value used
68 to convert the extra C rendered extractable by fumigation to biomass-C (Jenkinson et
69 al., 2004; Joergensen et al., 2011). In recent times, the findings of several studies that
70 investigated the impact of various interfering factors, such as soil physicochemical
71 properties (e.g. pH, moisture content, SOM) and environmental factors (e.g. drying–
72 wetting episodes, temperature changes, fresh labile-C substrate addition), or interacting
73 compounds (e.g. black carbon) on the extraction process and its efficiency (e.g., Ross,
74 1989; Haney et al., 2001; Durenkamp et al., 2010) have raised concerns about the
75 robustness of using a fixed k_{EC} value for wide-ranging applications. Further, several of
76 the studies conducted to assess the influence of methodology artefacts, such as
77 extraction modalities – time and shaking conditions, and concentration and type of
78 extractants, also reached differing conclusions (see Tate et al., 1988; Ross, 1989;
79 Sparling and West, 1989; Couteaux et al., 1990; Ross, 1990; Sparling et al., 1990;
80 Ross, 1992; Dictor et al., 1998; Haubensak et al., 2002; Durenkamp et al., 2010; Alessi
81 et al., 2011; Jost et al., 2011).
82 Apart from the interference and methodology-related issues, argument has continued
83 on the appropriateness of using a fixed k_{EC} value to convert C-flush to biomass-C when
84 working with different soil types or on the same soil with different treatments (Bremer
85 and Kuikman, 1994; Haubensak et al., 2002; Boucard et al., 2008; Alessi et al., 2011).
86 Partly because the technique was originally developed for research in agriculture and
87 land use, there are only a handful of studies that have assessed the effect of the
88 presence of contaminants (organic or metals) in soil on the extraction efficiency by the
89 FE technique (Dumontet and Mathur, 1989; Barajas Aceves et al., 1999; Dawson et al.,

90 2007; Durenkamp et al., 2010). However, most of the studies on contaminated soils
91 have used various k_{EC} values without considering the interfering influences of the
92 contaminants on the extraction efficiency. Consequentially, if the presence of a given
93 contaminant can influence the extraction efficiency, the choice of an appropriate k_{EC}
94 value for the conversion of C-flush to biomass-C in a soil becomes important. This, to
95 a large extent, will affect the quantification of biomass-C as well as the accuracy and
96 interpretations of models use to elucidate the fate and behaviour of carbon substrates
97 and their turnover in contaminated soils. In addition, the concentration of such
98 contaminants may also exert some appreciable influence, further dictating which k_{EC}
99 value is appropriate. This may become complicated as previous studies have showed
100 that soil type and depth can affect the estimation of k_{EC} (Gregorich et al., 1991).
101 Therefore, this paper aimed to assess the influence of increasing diesel concentration
102 on the quantification of biomass-^{14}C by FE technique in soils with differing properties.
103
104 **2. Materials and methods**
105 *2.1. Materials*
106 Non-labelled glucose (purity >99%) and [1-^{14}C]glucose (specific activity 55.7 mCi
107 mmol^{-1}, radiochemical purity >99.6%) were purchased from Sigma–Aldrich, UK.
108 Diesel oil was obtained from a UK fuel station in Lancaster, UK (specific gravity 0.85,
109 C-content 87%; information from supplier).
110
111 *2.2. Microcosm set-up and incubation*
112 Four soils, differing in their physicochemical and microbiological characteristics, were
113 sampled (A horizon; 5–20cm) from various locations in Lancashire, UK. The grid
114 references and properties of the soils are presented in Table 1. Prior to the start of

115 experiment, the sieved soil samples (≤2 mm) were conditioned at 21 ± 1 °C for 10 d to
116 minimum the priming effect and allow microbial respiration to stabilise (Kemmitt et
117 al., 2008). The soils were amended by one step spiking/rehydrating procedure as
118 described by Doick et al. (2003). Different amounts of diesel oil were added to soil to
119 give concentrations of 0, 10, 100, 1000 and 5000 mg kg^{-1}. Then, sufficient amount of
120 pre-sterilised minimal basal salt (MBS) solution (Fenlon et al., 2011) containing non-
121 labelled (108 μg ^{12}C g^{-1} DW) and radiolabelled glucose (*ca.* 40 Bq g^{-1} DW) was added
122 to bring the final moisture content of the soils to 60% of their respective water holding
123 capacities. Following amendment, portions of the control and amended soils (20 ± 0.2
124 g) placed in modified 250 ml Schott bottles (Reid et al., 2001) were incubated under
125 static conditions, in triplicates, for 35 d at 21 ± 1 °C in the dark. The radiorespirometric
126 assays were setup to monitor $^{14}CO_2$ evolution continuously while ^{14}C-biomass
127 quantification followed the FE technique (Vance et al., 1987) after 5, 15 and 30 d of
128 incubation.
129
130 *2.3. Soil-associated ^{14}C-activity in amended soils*
131 At each sampling time (0, 5, 15 and 30 d), a portion of the amended soil (1 g DW) was
132 oxidized (Packard 307 sample oxidizer; Canberra Packard, UK) to determine the ^{14}C-
133 activity remaining in the respirometers (i.e. soil-associated residual ^{14}C). The efficiency
134 of ^{14}C-activity recovery determined prior to sample oxidation was ≥97%, at all times.
135 The ^{14}C-activity was measured by liquid scintillation counting (LSC) (Tri-Carb
136 2300TR liquid scintillation counter; Canberra Packard, Belgium) after storage in the
137 dark for 24 h to normalise the effects of chemo-luminescence.
138

139 *2.4. Quantification of soil microbial biomass-^{14}C and calculation of variable k_{EC}*

140 All parameters reported in the text were expressed as percentages of the initial ^{14}C-
141 activity ($^{14}C_{init}$) added. ^{14}C-glucose incorporated as ^{14}C-biomass was derived from the
142 equation (Vance et al., 1987; Sparling et al., 1990):

143 $$^{14}C\text{-Biomass (fixed)} = {}^{14}C\text{-flush}/k_{EC}, \quad (1)$$

144 where, $$^{14}C\text{-flush} = {}^{14}C_{\text{fumigated soil}} - {}^{14}C_{\text{unfumigated soil}} \quad (2)$$

145 and a fixed k_{EC} (0.35) was used (Sparling et al., 1990).

146 It was assumed that ^{14}C-activity not respired as $^{14}CO_2$ during mineralisation or readily
147 extractable by K_2SO_4 in unfumigated soil sample was incorporated as ^{14}C-biomass with
148 negligible amount left as extracellular non-microbial ^{14}C-metabolites (Sparling and
149 West, 1988; Bremer and van Kessel, 1990; Sparling et al., 1990; Dictor et al., 1998);
150 thus, ^{14}C-biomass was recalculated as (Sparling and West, 1988):

151 $$^{14}C\text{-Biomass (variable)} = [(^{14}C_{init}) - (^{14}CO_2) - (^{14}C_{\text{unfumigated soil}})] \quad (3)$$

152 with corresponding variable k_{EC} derived as:

153 $$\text{Variable } k_{EC} = {}^{14}C\text{-flush}/\ {}^{14}C\text{-biomass (variable)} \quad (4)$$

154

155 *2.5. Statistical analysis*

156 Multivariate analysis of variance (MANOVA) was performed by General Linear
157 Model (GLM) program using the SPSS 19 software for Windows (IBM SPSS Inc.,
158 USA). Diesel concentration and soil type were factored as between-subject
159 independent variables while incubation time was used as within-subject covariate.
160 Where necessary, Tukey's LSD tests were used for post-hoc comparisons of means (P
161 ≤ 0.05).

162

3. Results and discussion

3.1. Temporal changes in soil-associated residual ^{14}C-activity

The initial rapid decline in the ^{14}C-activity added for the first 3 d in the soils (data not shown) was due mainly to the rapid $^{14}CO_2$ efflux (Bremer and Kuikman, 1994; Nguyen and Guckert, 2001). By 5 d of incubation, the ^{14}C-activity left as residual ^{14}C ranged from 56 to 68% in the control soils, from 55 to 68% in the 100 mg kg^{-1} soils, and from 54 to 62% in the 5000 mg kg^{-1} soils, respectively (Figure 1). After 30 d of incubation, amounts of residual ^{14}C have decreased to 44–56% in the control soils, 40–58% in the 100 mg kg^{-1} soils, and 27–49% in the 5000 mg kg^{-1} soils, respectively. Diesel, at the higher concentrations (1000–5000 mg kg^{-1}) had effect in some of the soils; however, multivariate analysis indicated that the overall trend in the removal of ^{14}C-activity was affected by the soil type ($P = 0.051$) but not by the concentration of diesel ($P > 0.05$) (Table 2). As expected, the residual ^{14}C were markedly affected by incubation time (Table 2; $P < 0.001$); this was as a result of the mineralisation of extracellular ^{14}C-metabolites and decomposition of the dead ^{14}C-biomass, though at much slower rate after the initial rapid mineralisation of the labile ^{14}C-glucose (Saggar et al., 1994).

3.2. Quantification of ^{14}C-biomass in diesel-amended soils by FE technique

Based on either a fixed value of 0.35 (Sparling et al., 1990) or the variable k_{EC} values obtained directly from the soils investigated (Table 3), ^{14}C-biomass was significantly affected by diesel concentration ($P < 0.001$), and generally differed between soil types ($P < 0.05$) and as incubation time increased ($P < 0.001$) (Table 2). However, when the fraction of ^{14}C-biomass in the residual ^{14}C-pool was considered, no clear trends were observed; ^{14}C-biomass accounted for *ca.* 43–111% or 70–112% of the residual ^{14}C if a fixed or variable k_{EC} value was used for the conversion, respectively (Figure 2). It is

188 remarkable that when calculated with a fixed k_{EC} value, the fraction of ^{14}C-biomass in
189 the residual ^{14}C-pool was significantly (P = 0.019) lower at the higher diesel
190 concentrations (≥1000 mg kg^{-1}) for all soils (P = 0.043) (Table 2; Figure 2A). By
191 contrast, when variable k_{EC} values were used, the fraction of ^{14}C-biomass in the
192 residual ^{14}C-pool was not affected by diesel concentration (P = 0.979) in any of the
193 soils (P = 0.306) (Table 2; Figure 2B). This indicates that the interpretation of the
194 effect of diesel concentration on microbial activity and biomass formation in soils will
195 be dependent on whether fixed or variable k_{EC} is used to quantify ^{14}C-biomass.
196 In a study of the effect of fuel oil on microbial biomass and activity it was suggested
197 that oil (as high as *ca.* 28.5 g kg^{-1}) had no significant effect on the K_2SO_4 extraction
198 and on the quantification of biomass-C after UV-persulfate oxidation by IR-detection
199 using a Dohrman DC 80 automated system (Joergensen et al., 1995). Meanwhile, as
200 observed in this present study too, it was acknowledged in the previous study that
201 schlieren and globules of fat formed on the surface of the K_2SO_4 extracts may be a
202 factor of interference for the C measurements (Joergensen et al., 1995). The difference
203 between the present finding and that of Joergensen et al. (1995) could be related to the
204 means of quantification. Since the automated IR-detection system used for
205 quantification (Wu et al., 1990) in their study cannot discriminate between oil-C, soil-C
206 or biomass-C, the influence of the high fuel oil concentration might have been greater
207 than suspected and gone undetected.
208 Comparatively, as incubation time increased the decrease in ^{14}C-biomass obtained with
209 variable k_{EC} values showed greater consistency than ^{14}C-biomass derived with the fixed
210 k_{EC} value, for all soil types (Table 2). This suggests that the choice of a k_{EC} value can
211 influence the pattern of change and the magnitude of the "active" ^{14}C-biomass fraction
212 in the total SOC pool of soils contaminated with diesel.

213

214 *3.3. Influence of diesel concentration on the FE technique*

215 Based on the assumptions used for the derivation of variable k_{EC} (Equations 3 and 4),
216 any influence of diesel on the propriety of K_2SO_4 extraction capacity (i.e. k_{EC}) will
217 affect the quantification of ^{14}C-biomass in soil. Although there were no marked
218 variations when the variable k_{EC} values obtained were compared between soil types (P
219 = 0.634), it appeared that diesel concentration has some influence (P = 0.059), which
220 was more pronounced as incubation time increased (P = 0.003; Table 2). Whilst the
221 variable k_{EC} values usually decreased with increasing diesel concentration, they were
222 significantly lower in all soils amended with diesel ≥1000 mg kg^{-1} (Table 3). This
223 indicates that high diesel concentrations in soil may undermine the appropriateness of
224 the use of a single 'conversion factor' as widely employed for quantification of ^{14}C-
225 biomass (Sparling et al., 1990; Joergensen et al., 2011). This submission is further
226 strengthened when a regression line was fitted between the ^{14}C-biomasses derived from
227 a fixed and the variable k_{EC} values (Figure 3). Though the correlation appeared strong
228 and linear (r^2 = 0.84; $P < 0.001$), the intercept (19.69%) and slope (0.57) indicated that
229 the relationship was not ideal (1:1). When the regression fit was forced to intercept at 0
230 (i.e. 1:1 slope), the correlation was weak (r^2 = 0.32; $P < 0.05$) and the other regression
231 models tested did not improve the fit (data not shown). However, the independent-
232 sample Student's t test indicated that the overall mean k_{EC} value of 0.37 obtained for all
233 the control soils used in this study (Table 4) is similar to the k_{EC} value of 0.35 proposed
234 by Sparling et al. (1990) or the same as 0.38 proposed in the original method by Vance
235 et al. (1987). The k_{EC} values that have been used in the literature ranged widely from
236 0.15 to 0.98 (see Sparling et al., 1981b; Sparling et al., 1981a; West et al., 1986;
237 Sparling and West, 1988; Tate et al., 1988; Gregorich et al., 1990; Sparling et al., 1990;

238 Bremer and Kuikman, 1994; Joergensen, 1996) with most of the studies adopting a k_{EC}
239 value without sound empirical basis. Several other investigators have cautioned the
240 arbitrary selection of k_{EC} value for the quantification of biomass-C (Bremer and
241 Kuikman, 1994; Haubensak et al., 2002; Boucard et al., 2008; Alessi et al., 2011).
242 After the K_2SO_4 extraction of both the unfumigated and fumigated soil samples, the
243 spent pellets were sample oxidised as previously described (data not shown). From the
244 data obtained, there was no clear indication of the influence of diesel on ^{14}C-carbon left
245 in the unfumigated samples; however, larger amounts the ^{14}C-carbon were recovered in
246 the fumigated soil samples with higher diesel concentrations. Apparently, diesel
247 exhibited similar effect as activated charcoal on the extraction efficiency of biomass-C.
248 It was observed that the presence of activated charcoal (as low as 3.5 mg C g^{-1})
249 significantly decreased the extraction efficiency of biomass-C, whereas biochar (as
250 high as 28 mg C g^{-1}) had negligible effect (Durenkamp et al., 2010). Because the
251 determination of biomass-C by FE technique was dependent on the type of black
252 carbon, on the concentration of K_2SO_4 solution, and on soil type, the authors advised
253 that each type of black carbon should be tested beforehand for its impact on the
254 determination of biomass by FE technique. The results of this present study also
255 question the credibility of applying a single k_{EC} value to all soils despite the potential
256 interference that the presence of other compounds can introduce to the extraction
257 process; hence, it is more appropriate to use *in-situ* derived k_{EC} rather than a fixed k_{EC}
258 value for contaminated soils. Several authors have presented other reasons why the use
259 of a fixed k_{EC} value could result in substantial errors in quantification of biomass-C
260 (West et al., 1986; Bremer and van Kessel, 1990; Ross, 1990; Bremer and Kuikman,
261 1994; Turner et al., 2001). Though, it has even been suggested that emphasis should be
262 placed on the relative differences within and between soils using data that have not

been converted to ^{14}C-biomass (West et al., 1986), it is still important to quantify the size of microbial biomass in soil.

3.4. Conclusions

In the soils investigated, diesel concentration above 1000 mg kg^{-1} had substantial influence on the quantification of ^{14}C-biomass by the FE technique. Hence, it is advised that except on the basis of comparison only, an *in-situ* derived k_{EC} value is more appropriate to quantify biomass-C in diesel oil-contaminated soils. Further studies using δ^{13}C-carbon and ^{14}C-carbon aged oil-contaminated soil and the procedures that can concurrently and differentially quantify C from the various sources, such as the organic contaminants and soil microbial biomass (see Murage and Voroney, 2007) may provide a more detailed understanding of the influence of increasing concentration of organic contaminants on the quantification of biomass-C by FE technique. It will also be valuable to evaluate the influence of various organic and metal contaminants with the possibility of obtaining a series of k_{EC} values for a better quantification and comparative analysis of biomass-C in soils depending on the dominant contaminant(s). Meanwhile, the objectives of this study did not include the calibration of a k_{EC} value for soils contaminated with diesel oil so no k_{EC} has been recommended.

Acknowledgments

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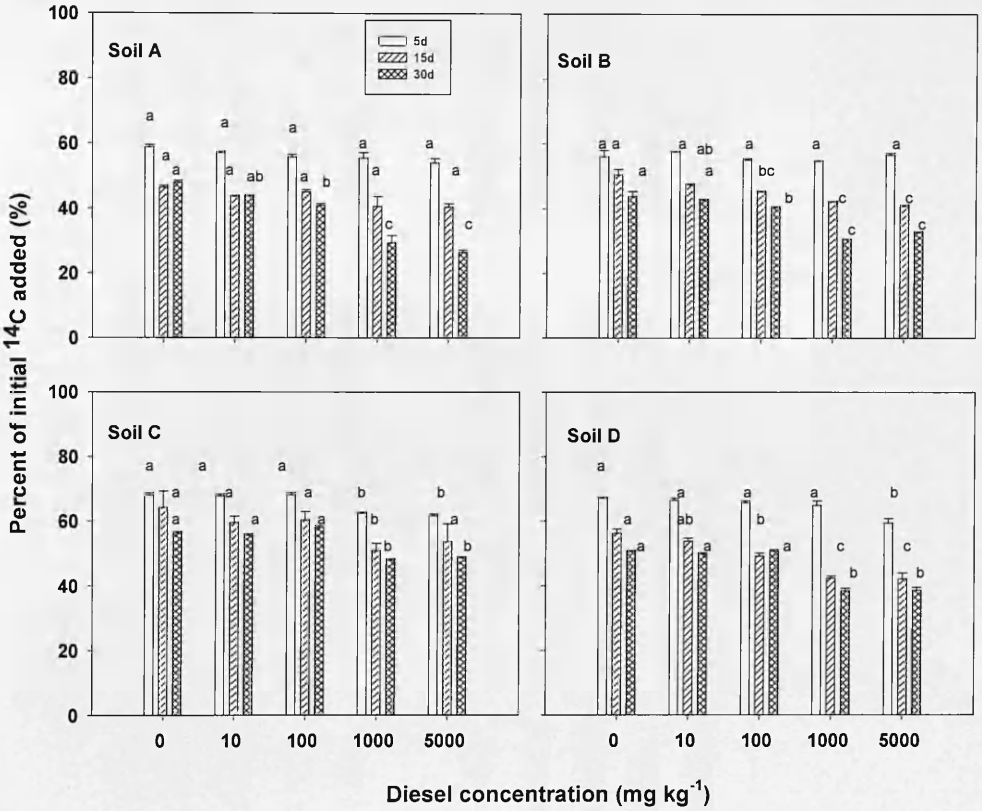
442

443 **Figures**

444



445

446 Figure 1: Soil-associated residual ^{14}C after 5, 15 and 30 d of incubation of unamended
447 and amended soils. Bars are the means ± SEM of 3 replicates. For any particular
448 incubation time (i.e. bars with the same pattern) different lower-case letters indicate
449 means ± SEM that are significantly different ($P < 0.05$).

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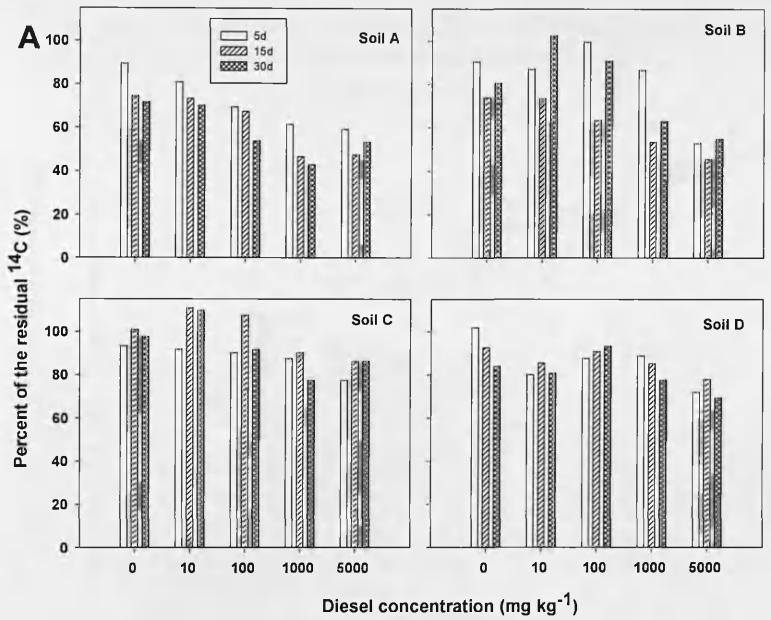

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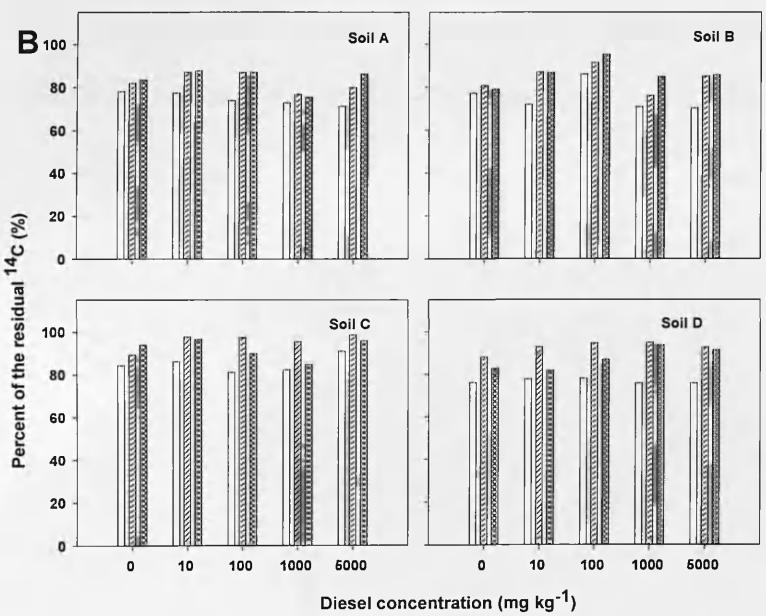

452

453 Figure 2: Percent of ^{14}C-biomass in the residual ^{14}C after 5, 15 and 30 d of incubation
454 of un-amended and diesel oil-amended soils. ^{14}C-biomass was based on (A) a fixed k_{EC}
455 of 0.35 and (B) variable k_{EC}. Bars are the means of 3 replicates.

456

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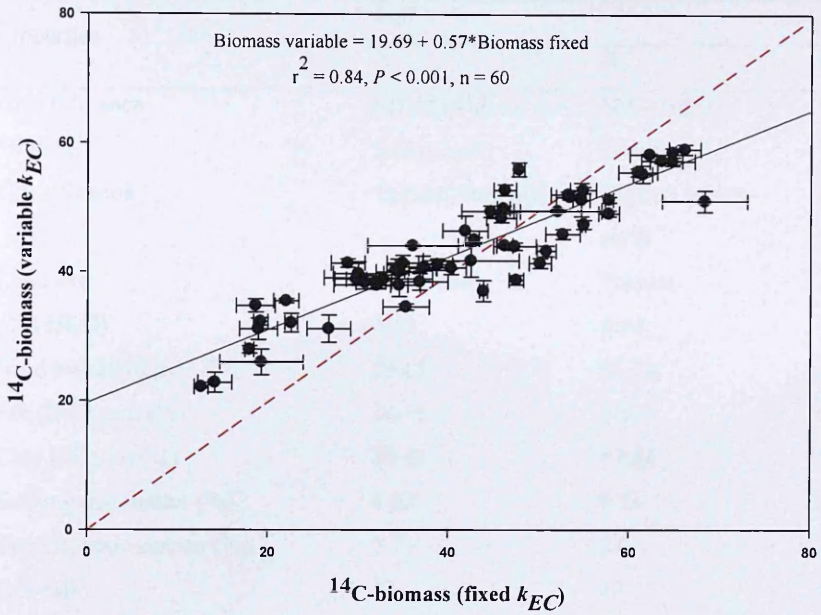

458

459 Figure 3: Correlation between ^{14}C-biomass calculated based on a fixed value or the

460 variable k_{EC} values. The dotted line is a regression fit forced to intercept at 0 ($r^2 = 0.32$,

461 $P < 0.05$)

Tables

Table 1: Physicochemical and microbiological properties of soils.

Properties	Soil			
	A	B	C	D
Grid reference	SD 496402	SD 491655	SD 511775	SD 447543
Texture	Sandy loam	Loam	Silty clay	Clay loam
Classification	Typical Stagnogley	Typical brown-earth	Earthy oligo-fibrous peat	Typical humic alluvial gley
Land use	Grassland	Pasture	Arable	Arable
pH (dH_2O)	6.53	5.44	7.50	6.93
Sand (60-2000 μm) (%)	55.63	55.39	10.45	38.77
Silt (2-60 μm) (%)	24.96	26.77	47.37	34.44
Clay (<2 μm) (%)	19.41	17.84	42.18	27.79
Soil organic matter (%)*	4.82	9.33	27.15	10.25
Total organic carbon (%)	1.7	3.0	19.5	3.5
C:N ratio	12	12	15	13
Extractable nitrate (mg kg^{-1})	489	641	1730	1318
Extractable P (mg kg^{-1})#	26.48	14.85	34.05	42.40
Available K (mg kg^{-1})	143	239	297	752
Available Mg (mg kg^{-1})	579	2503	1523	2249
Fungal/Bacterial biomass-C ratio	1.31	1.09	1.02	1.25
Bacterial counts (x 10^3 CFU g^{-1})	363	49	2590	307
qCO_2 (μg mg^{-1} biomass-C h^{-1})[a]	5.02	5.22	3.86	4.72
RQ (μg CO_2 μg^{-1} O_2)[b]	1.10	1.00	1.23	1.25

* Loss on ignition; # Olsen's bicarbonate method; [a] qCO_2: metabolic quotient; [b] RQ: respiratory quotient

Table 2: Multivariate analysis for the effects of soil type (A, B, C, D), diesel concentration (0, 10, 100, 1000, 5000 mg kg^{-1}) and incubation time (5, 15, 30 d) on soil-associated residual ^{14}C, ^{14}C-biomass, variable k_{EC}, and ^{14}C-biomass/residual ^{14}C.

Variables		Residual ^{14}C		^{14}C-biomass (fixed)		^{14}C-biomass (variable)		k_{EC} (variable)		^{14}C-biomass/residual ^{14}C (fixed)		^{4}C-biomass/residual ^{14}C (variable)	
	Df	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Main effect													
ST	3	3.078	**0.051**	7.819	**0.001**	57.435	**< 0.001**	0.582	0.634	3.269	**0.043**	1.287	0.306
DC	4	0.266	0.896	4.260	**0.012**	3.002	**0.043**	2.716	**0.052**	3.761	**0.019**	0.106	0.979
IT	1	80.639	**<0.001**	46.910	**< 0.001**	235.286	**< 0.001**	11.165	**0.003**	3.050	0.096	6.159	**0.022**
Two-way													
ST x DC	12	0.131	1.000	0.265	0.989	0.869	0.588	0.325	0.975	0.436	0.929	0.159	0.999
ST x IT	3	0.577	0.637	0.952	0.434	2.336	0.104	0.846	0.485	1.285	0.307	0.018	0.997
DC x IT	4	1.429	0.261	1.283	0.310	6.971	**0.001**	0.941	0.461	1.277	0.312	0.083	0.987
Three-way													
ST x DC x IT	12	0.171	0.998	0.157	0.999	0.975	0.502	0.198	0.997	0.222	0.995	0.230	0.994
Residuals	20												

[1] Soil type (ST) and diesel concentration (DC) are fixed factors while incubation time (IT) is a covariate

469 Table 3: Influence of diesel concentration and incubation time on estimations of

470 variable k_{EC} in four UK soils.

Incubation (d)	Concentration (mg kg^{-1})	Variable k_{EC}[§]			
		A	B	C	D
5	0	$0.40 \pm 0.01^{a\dagger}$	0.41 ± 0.03^{a}	0.39 ± 0.01^{a}	0.47 ± 0.02^{a}
	10	0.37 ± 0.00^{b}	0.42 ± 0.01^{a}	0.37 ± 0.01^{a}	0.36 ± 0.01^{b}
	100	0.33 ± 0.01^{c}	0.40 ± 0.00^{a}	0.39 ± 0.01^{a}	0.39 ± 0.01^{b}
	1000	0.30 ± 0.01^{d}	0.43 ± 0.01^{a}	0.37 ± 0.01^{a}	0.41 ± 0.02^{ab}
	5000	0.29 ± 0.00^{d}	0.26 ± 0.00^{b}	0.30 ± 0.01^{b}	0.33 ± 0.01^{b}
15	0	0.32 ± 0.01^{a}	0.32 ± 0.01^{a}	0.40 ± 0.03^{a}	0.37 ± 0.01^{a}
	10	0.30 ± 0.01^{a}	0.30 ± 0.04^{ab}	0.39 ± 0.00^{a}	0.32 ± 0.01^{a}
	100	0.27 ± 0.01^{ab}	0.24 ± 0.04^{ab}	0.39 ± 0.02^{a}	0.32 ± 0.02^{a}
	1000	0.21 ± 0.01^{b}	0.24 ± 0.01^{ab}	0.33 ± 0.04^{a}	0.29 ± 0.02^{b}
	5000	0.21 ± 0.02^{b}	0.19 ± 0.01^{b}	0.31 ± 0.03^{a}	0.29 ± 0.02^{b}
30	0	0.30 ± 0.02^{a}	0.36 ± 0.03^{ab}	0.37 ± 0.03^{a}	0.36 ± 0.06^{a}
	10	0.28 ± 0.02^{ab}	0.42 ± 0.03^{a}	0.38 ± 0.01^{a}	0.35 ± 0.02^{a}
	100	0.22 ± 0.01^{b}	0.33 ± 0.01^{ac}	0.36 ± 0.01^{a}	0.38 ± 0.02^{a}
	1000	0.20 ± 0.02^{b}	0.26 ± 0.02^{bc}	0.32 ± 0.01^{a}	0.27 ± 0.01^{a}
	5000	0.22 ± 0.01^{b}	0.22 ± 0.02^{c}	0.32 ± 0.02^{a}	0.30 ± 0.04^{a}

471 [§] Values are means ± standard errors of three replicates

472 [†] Different lower-case letters down the column for each sub-group of "Incubation" indicate means that are statistically different (P
473 < 0.05).

474

475 Table 4: Least square means of variable k_{EC} values for soil type (A, B, C, D), diesel
476 concentration (0, 10, 100, 1000, 5000 mg kg^{-1}) and incubation time (5, 15, 30 d).

Soil type		Diesel concentration (mg kg^{-1})		Incubation time (d)	
Group	Mean	Group	Mean	Group	Mean
A	0.280[a†]	0	0.371[a]	5	0.370[a]
B	0.321[ab]	10	0.354[a]	15	0.300[b]
C	0.358[b]	100	0.335[b]	30	0.310[b]
D	0.347[b]	1000	0.303[c]		
		5000	0.270[d]		
SE[‡]	0.00533		0.00596		0.00461

477 [‡]Standard error of LS Mean for each group
478 [†]Different lower-case letters down the column for each group of variables indicate means that are statistically different ($p < 0.05$).
479

480

481

Paper XI

Short-term microbial turnover of labile carbon in diesel oil-contaminated soils: influences of diesel concentration and soil texture

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Abstract

The effects of diesel oil concentrations (0–5000 $mg_{oil\text{-}C}$ kg^{-1}_{soil}) on the short-term turnover of labile carbon substrate were evaluated in four contrasting soil types. Uptake and mineralisation of ^{14}C-glucose and formation of new microbial ^{14}C-biomass were monitored over 35-d incubation. The cumulative $^{14}CO_2$ respired accounted for 39 to 63% of the ^{14}C-glucose input to the control soils and was influenced by clay contents. The effect of diesel concentration followed similar trends in all soils: extent of $^{14}CO_2$ respired, biophysical quotient (BQ) and metabolic quotient ($q^{14}CO_2$) increased while ^{14}C-biomass size, microbial yield coefficient (*Yc*) and microbial ^{14}C-turnover time (MTT) decreased with increasing diesel concentration. The *Yc* was higher and the MTT increased but $q^{14}CO_2$ decreased as the amounts of SOM and clay increased in soils. Collectively, the results indicate that the extant soil microbiota expend more energy for maintenance requirements as diesel concentration increased in soils; though the effect was less in the heavy-textured soil.

Capsule:

Diesel concentration and soil texture affect the turnover rate of labile carbon substrate in soil.

Keywords: *Carbon turnover; Microbial activity; Microbial biomass; Metabolic efficiency; Mineralisation; Petroleum oil*

Highlights:

- *Effect of diesel concentration on short-term turnover of labile C monitored in soils.*
- *$^{14}CO_2$ and $q^{14}CO_2$ increased with increasing diesel concentration.*
- *^{14}C-biomass and ^{14}C-turnover time decreased with increasing diesel concentration.*
- *Effect of diesel was less significant as amount of SOM and clay increased in soil.*
- *Diesel concentration, SOM and clay contents influenced turnover of labile C*

1. Introduction

Crude petroleum and its derived oils, such as diesel oil, contain complex mixtures of hydrophobic organic contaminants – asphaltenes, aliphatic hydrocarbons and polycyclic aromatic hydrocarbons (PAHs) which are potentially recalcitrant and accumulate in the environment (Wang and Bartha, 1990). These organic contaminants serve as food to only a small fraction of soil microbial populations with specialised enzymatic capability, and are toxic for many soil microbiota causing notable physiological changes including alterations in the metabolic activity (Mueller et al., 2003). The impacts of diesel on soil microorganisms have been extensively investigated with respect to soil ecology and ecotoxicology as well as bioremediation (Aldaya et al., 2006; Coulon et al., 2010; Gandolfi et al., 2010; Tejada and Masciandaro, 2011). Most of these studies are focused on the fate and behaviour of the organic contaminants, and on the development of catabolic abilities within the microbial communities to degrade or detoxify the contaminants (Hickman et al., 2008; Liu et al., 2008; Rhodes et al., 2008).

There is a large collection of studies in the literature on the effects of various carbon-composing amendments to improve microbial degradation of organic contaminants with evidence pointing to an enhancing effect of the labile carbon substrates, especially when sufficient amounts of other requisite nutrients like nitrogen and phosphorus are present (e.g. Liebeg and Cutright, 1999; Yu et al., 2005; Taccari et al., 2012; Xu and Lu, 2012; Lladó et al., 2013; Pelaez et al., 2013; Tejeda-Agredano et al., 2013). In comparison, however, there are fewer numbers of studies on the effects of organic contaminants on microbial metabolism and turnover of labile carbons in soil; in particular, the effect of contaminant concentration has been given little attention. In effect, such studies will provide for a better understanding of the role and the fate of

68 labile carbon substrates during attenuated or enhanced biodegradation of petroleum
69 hydrocarbons in soils.
70 There is substantial evidence in the literature that soil characteristics, such as clay
71 content and mineralogical composition can influence the decomposition and stability of
72 labile carbon substrates in soil (e.g. Sorensen, 1975; Saggar et al., 1994; Saggar et al.,
73 1996; Saggar et al., 1999). However, virtually all of these studies were on
74 "uncontaminated" soils; information on the interfering or interacting effects of soil
75 texture and the presence of organic contaminants, especially petroleum hydrocarbons,
76 remains scarce. Bardgett and Saggar (1994) examined the effects of heavy metal
77 contamination on the short-term decomposition of ^{14}C-glucose in a pasture soil. The
78 authors suggested that the presence and increasing concentration of chromium, copper
79 and arsenic caused a significant decrease in the capability of indigenous soil
80 microorganisms to utilise labile carbon substrates for biomass synthesis, resulting in
81 more energy been diverted for maintenance requirements. Heavy metals are not known
82 to be degradable by microorganisms, and evidence of their involvement in microbial
83 metabolic process is mainly of inhibition and/or toxicity (Thavamani et al., 2012; Peng
84 et al., 2013). Whereas, most organic contaminants are biodegradable, at least to certain
85 extents, they may also exhibit inhibitory and/or toxic effects during microbial
86 metabolism (Lors et al., 2011). Hence, it is not yet clear whether organic contaminants,
87 such as petroleum hydrocarbons, will exhibit similar effects as the heavy metals.
88 Therefore, this study was conducted to determine the short-term effect of diesel oil
89 concentration on the microbial utilisation efficiency and turnover of glucose through
90 microbial mineralisation and biomass formation. The influence of the soil clay and
91 organic matter contents on these effects were also assessed using soils with differing
92 clay and organic matter contents.

2. Materials and methods

2.1. Chemicals

Non-labelled (purity >99%) and [1-^{14}C]glucose (55.7 mCi mmol^{-1}, radiochemical purity >99.6%) were purchased from Sigma–Aldrich (Poole, UK). Diesel oil (specific gravity 0.85, C-content 87%) was obtained from a local BP fuel station in Lancaster, UK. Chemicals for the minimum basal salts (MBS) solution were supplied by BDH Laboratory Supplies and Fisher Chemicals, UK.

2.2. Soil physicochemical and microbiological properties

Four soils were collected (A horizon; 5–20 cm) from various locations in the UK (Table 1); their textural profiles and organic matter contents were characterised as reported by Rhodes et al. (2010). Microbiological characterisation of the soils was carried out just before the start of this experiment. Microbial respiratory quotient (RQ: ratio of CO_2 production to O_2 consumption) and biomass-C of soils were quantified by measuring the respiration rates in an aerobic closed static system on a MicroOxymax respirometer (Multiple Sensor O_2/CO_2 10-Chamber System, Columbus) (Towell et al., 2011). Fungal–to–bacterial biomass-C ratio was estimated using the substrate-induced respiratory (SIR) method (Anderson and Domsch, 1978) combined with selective inhibition techniques (Nakamoto and Wakahara, 2004). Oxytetracycline hydrochloride (4 mg g^{-1}) and cycloheximide (2 mg g^{-1}) were used as bacterial and fungal inhibitors, respectively. Culturable heterotrophic and PAH-degrading bacterial cells were enumerated by the standard spread plate method (Lorch et al., 1995). Background concentrations of total petroleum (aromatic and aliphatic) hydrocarbons (TPH) were quantified by shake-extraction of samples in 1:1 solution of dichloromethane/acetone

for 24 h. Extracts were passed through alumina column to clean up and separate the aliphatic and aromatic components. Quantitative analysis was carried out by capillary gas chromatography coupled with flame ionisation detection (GC-FID), essentially following the EPA Method 8015 (US EPA, 1987).

2.3. Microcosm set-up and microbial utilisation of ^{14}C-glucose in diesel-amended soils

Soils were air dried at room temperature for 48 h to *ca.* 40% of their respective water holding capacities (WHCs), sieved (≤2 mm) and stored at 4 °C until required. Prior to the start of experiments, the sieved soil samples were conditioned at 21 ± 1 °C in the dark for 10 d to allow microbial activity to stabilise (Kemmitt et al., 2008). Different amounts of diesel oil were amended to soil sub-samples (500 g) following the procedure described by Doick et al. (2003) to final nominal concentrations of 10, 100, 1000 and 5000 $mg_{oil\text{-}C}$ kg^{-1} soil. Sufficient amounts of pre-sterilised minimal basal salts (MBS) solution (Fenlon et al., 2011) containing non-labelled glucose (108 μg C g^{-1}) and radiolabelled glucose (*ca.* 40 Bq g^{-1}) were placed in stainless containers to bring the final moisture content of the soils to 60% of their respective WHC. Then, the amended soil (50–100 g at a time) was gradually added to the solution and thoroughly mixed using stainless steel spatula. The MBS solution contained both N and P sources to ensure that these nutrients were not limiting microbial growth. Control soil was mixed with ^{14}C-glucose-MBS solution without diesel. Blank samples were prepared with control soils mixed with MBS solution without ^{14}C-glucose for analytical corrections of background radioactivity in soils.

Following the amendments, soils (20 ± 0.2 g) were placed in modified 250 ml Schott bottles with Teflon®-lined screw caps, in triplicates. The radiorespirometric assay was incubated at 21 ± 1 °C under static conditions, and sampled at regular intervals for 35 d

143 (Bardgett and Saggar, 1994; Saggar et al., 1999). At 5, 15 and 30 d of incubation, ^{14}C-
144 biomass formed was determined by the chloroform fumigation-extraction (CFE)
145 procedure (Vance et al., 1987). This method is applicable to ^{14}C-spiked soils as the
146 newly synthesized microbial biomass could be estimated by fumigation-extraction
147 when 5 days or longer had passed since the substrate addition (Marstorp and Witter,
148 1999) and has been used to study the turnover of carbon substrates in soil (Bardgett and
149 Saggar, 1994; Bremer and Kuikman, 1994; Saggar et al., 1999). At each sampling time,
150 soil sample (1 g) was oxidized to determine the ^{14}C-activity remaining (i.e. soil-
151 associated residual ^{14}C-activity) using a Packard 307 sample oxidizer (Canberra
152 Packard, Berkshire, UK) (Boucard et al., 2008). The efficiency of ^{14}C-activity recovery
153 determined prior to sample oxidation was ≥97%, at all times. The ^{14}C-activity was
154 measured by liquid scintillation counting (LSC) (Tri-Carb 2300TR liquid scintillation
155 counter; Canberra Packard, Belgium) after storage in the dark for 24 h to normalise the
156 effects of chemo-luminescence.
157
158 *2.4. Model fit to mineralisation data, calculations and statistical analysis*
159 A biexponential model is used to describe microbial utilisation of labile ^{14}C-carbon
160 substrates in soils (Saggar et al., 1999; Glanville et al., 2012). The model was fitted by
161 a nonlinear regression GLM program run on SigmaStat version 3.5 (Systat Software
162 Inc., Chicago, IL). The equation consists of two first-order kinetic stages describing the
163 rapidly mineralising fraction followed by a second more slowly mineralising fraction:
164 $$A = A\cdot[1 - \exp\cdot(-k_r\cdot t)] + B\cdot[1 - \exp\cdot(-k_s\cdot t)] \quad (1)$$
165 where A = rapidly ^{14}C-mineralising fraction (labile); B = slowly ^{14}C-mineralising
166 fraction (stable); k_r and k_s are the first-order rate constants (h^{-1}) in the rapidly and
167 slowly mineralising fractions, respectively; and t is time in h.

168 A fixed k_{EC} value was not used for the conversion of ^{14}C-flush to ^{14}C-biomass because
169 of the likely interference diesel concentrations might have on the fumigation-extraction
170 efficiency. Instead, a mass balance equation was used to determine ^{14}C-biomass based
171 on the assumption that ^{14}C-glucose not respired as $^{14}CO_2$ during mineralisation or
172 readily-extractable by K_2SO_4 in the non-fumigated soil (which represents the portion
173 present as non-biomass ^{14}C-residues), had been immobilised as ^{14}C-biomass with
174 negligible amount of extracellular non-microbial metabolites (Sparling et al., 1990):

175 ^{14}C-Biomass = (^{14}C-glucose) – ($^{14}CO_2$) – (^{14}C-activity in unfumigated soil) (2)

176 To assess the microbial metabolic activity, the specific respiratory activity or metabolic
177 quotient [$q^{14}CO_2$; μg $^{14}CO_2$-C (mg ^{14}C-biomass)$^{-1}$ h^{-1}] was calculated as the ratio of
178 mineralisation rate (μg $^{14}CO_2$-C g^{-1} h^{-1}) to the ^{14}C-biomass (mg ^{14}C g^{-1}).

179 To evaluate the stabilisation effect of clay on labile carbon substrate, the biophysical
180 quotient (BQ) was used, and expressed as (Saggar et al., 1994; Saggar et al., 1999):

181 BQ = [($\sum^{14}CO_2$)/(^{14}C-biomass + non-biomass ^{14}C-residues) (3)

182 To evaluate the labile carbon utilisation efficiency, the yield coefficient (*Yc*) was
183 derived as:

184 Yc = [($\sum^{14}CO_2$)/($\sum^{14}CO_2$ + ^{14}C-biomass) (4)

185 Two independent methods were used to estimate microbial ^{14}C-turnover in soil. First,
186 an empirical equation using the data for rates and extents of mineralisation, biomass
187 formation and ^{14}C-glucose utilisation efficiency (Cheng, 2009; Blagodatskaya et al.,
188 2011):

189 Microbial turnover time (MTT) = [^{14}C-biomass·(1–Yc)/Yc]/(R_s–^{14}C-biomass ·R_m) (5)

190 where, R_s is mineralisation rates (μg $^{14}CO_2$-C g^{-1} h^{-1}) at 5 and 30 d, and R_m is the
191 microbial maintenance respiration rate estimated as 0.0033% of ^{14}C-biomass h^{-1}
192 (Anderson and Domsch, 1985).

193 The second, mean residence time (MRT) was calculated using the parameters derived
194 from fitting a biexponential model (similar to Eq. (1)) to the residual ^{14}C data, as an
195 empirical equation (Saggar et al., 1999):

196 $$MRT = A \cdot k_1 + (100 - A) \cdot k_2/(100 \cdot k_1 \cdot k_2) \quad (6)$$

197 where, A is the asymptotic residual ^{14}C (%), and k_1, and k_2 are rate constants (h^{-1}) for
198 rapidly and slowly mineralisable ^{14}C-residual pools, respectively.
199 Multivariate analysis of variance (MANOVA) was employed to evaluate the effects of
200 soil properties, diesel concentrations and incubation times on ^{14}C-glucose utilisation
201 using SPSS 19 software for Windows (IBM SPSS Inc., MA, USA). Where necessary,
202 Tukey's LSD tests were used for post-hoc comparisons of means ($P \leq 0.05$).
203

204 **3. Results**

205 *3.1. Soils*

206 The soils are representatives of major UK soil groups (e.g., typical Stagnogley, typical
207 brown earth, earthy oligo-fibrous peat and typical alluvial gley) (Avery, 1980) and
208 were under different land use at the time of sampling in the autumn of 2011.
209 Myerscough and Nether-Kellet soils are grasslands while Holme and Thurnham soils
210 are arable lands. The clay contents (178–422 g kg^{-1}), SOM (5–27%) and pH (5.44–
211 7.50) were very variable in the soils (Table 1). In the context of this manuscript, based
212 on the relative quantities of clay–SOM contents in the soils, Myerscough, Nether-
213 Kellet, Holme and Thurnham soils can be described as Low–Low, Low–Medium,
214 High–High and Medium–Medium, respectively. Microbial biomass-C (1072–2344 mg
215 C kg^{-1}), and the bacterial populations (4.9–259 x 10^4 CFU g^{-1}) in the soils differed
216 appreciably ($P < 0.05$); however, the fungal–bacterial biomass-C ratio (F/B ratio: 1.02–
217 1.31), metabolic quotient (qCO_2: 3.86–5.22 μg mg^{-1} biomass-C h^{-1}) and respiratory

quotients (RQ: 1.00–1.25 μg CO_2 μg^{-1} O_2) were comparable ($P > 0.05$). The background levels of petroleum hydrocarbons (∑16 PAHs: 30–103 μg kg^{-1}) in the soils were negligible compared to the amounts of diesel oil applied.

3.2. Mineralisation of ^{14}C-glucose

Throughout the 35-d incubation, the percent recovery of ^{14}C-activity ranged between 92 and 104% of the added ^{14}C-glucose, and was not affected by either diesel concentration or soil type. The rate of loss of ^{14}C-glucose was greatest during the first 2 to 4 h of the incubation (Figure 1; Table S1). The lag phases (amount of time before mineralisation reaches 5%) and the initial maximum rates (% h^{-1}) varied significantly ($P < 0.05$) between soils amended at the same diesel concentration but the effects of diesel concentration on these mineralisation indices were not significant ($P > 0.05$) (Table S1). In comparison between soil types, for the first 4 h, mineralisation rates were fastest in the high-clay Holme soil; however, between the 8 and 24 h, mineralisation rates have declined rapidly by a factor of 44 in the Holme soil, while on the average, the rates declined by only a factor of 4 in the other three soils with lower clay contents. Thereafter, the mineralisation rates declined further until 3 d, and reached an averaged steady rate of 0.01% h^{-1}, 0.04% h^{-1} and 0.06% h^{-1} after 10 d in the control soils, 100 $mg_{oil\text{-}C}$ kg^{-1} soils and 5000 $mg_{oil\text{-}C}$ kg^{-1} soils, respectively. The effects of diesel on mineralisation rates along the concentration gradient were particularly evident ($P < 0.05$) after 2 to 3 d of incubation (Figure 1); apparently well after the rapidly-mineralisable (labile) ^{14}C-pool would have become exhausted.

After 35 d of incubation, the extent of ^{14}C-glucose mineralisation differed between soils, and was dependent on the diesel concentration in the soils. For example, the extent of mineralisation was highest (53%) in the low-clay Nether-Kellet soil and

243 lowest (31%) in the high-clay Holme soil. The extent of mineralisation ranged from 31
244 to 53% in all control soils, from 31 to 54% in all the 100 $mg_{oil\text{-}C}$ kg^{-1} soils, and from 39
245 to 63% in all the 5000 $mg_{oil\text{-}C}$ kg^{-1} soils (Figure 1, Table S1). In general for all soil
246 types, the extents of mineralisation were significantly ($P < 0.05$) higher in the ≥1000
247 $mg_{oil\text{-}C}$ kg^{-1} soils relative to the control soils. Collectively for the control soils, there
248 was a negative, linear and highly significant correlation between the clay content and
249 the extents of mineralisation ($r^2 = 0.983$, $P = 0.009$). However, diesel apparently
250 interfered with the influence of clay on the extents of mineralisation; for example, the
251 correlations were less ($r^2 = 0.975$, $P = 0.030$) for the 1000 $mg_{oil\text{-}C}$ kg^{-1} soils and ($r^2 =$
252 0.934, $P = 0.033$) for the 5000 $mg_{oil\text{-}C}$ kg^{-1} soils, respectively.
253
254 *3.3. Microbial ^{14}C-biomass and non-biomass ^{14}C-residues*
255 The fractions of added ^{14}C-glucose remaining as stabilised ^{14}C-residues (i.e. ^{14}C-
256 biomass and non-biomass ^{14}C-residues) after 35 d incubation were influenced by both
257 clay and diesel concentrations (Figure 2). There were positive, linear and significant
258 correlations ($r^2 \geq 0.930$, $P < 0.05$) between clay content and ^{14}C-biomass in both
259 control and amended soils. For example, the soils having low clay contents (i.e.
260 Nether-Kellet and Myerscough) had comparatively lower ^{14}C-biomass (43 and 46%,
261 respectively) than the high-clay Holme soil (57%) at 5 d incubation. In terms of the
262 reduction in ^{14}C-biomass size as incubation time increased, it was pronounced in light-
263 textured soils (35 and 40%, respectively) than in the high-clay soil (53%) after 30 d. In
264 any particular soil type, increasing the concentration of diesel resulted in smaller
265 amounts of ^{14}C-actvivty incorporated to ^{14}C-biomass; this being significant ($P < 0.05$)
266 in the 1000 and 5000 $mg_{oil\text{-}C}$ kg^{-1} soils. For instance, after 5 d of incubation, ^{14}C-
267 biomass was 51, 49 and 45% and was 42, 38 and 31% after 30 in the control, 1000 and

268 5000 $mg_{oil\text{-}C}$ kg^{-1} Thurnham soils, respectively. Further, as incubation time increased,
269 ^{14}C-biomass reduced as diesel concentration increased in soils; this being significant (P
270 < 0.05) in the 1000 and 5000 $mg_{oil\text{-}C}$ kg^{-1} soils. Collectively for all soil types, ^{14}C-
271 biomass reduced from 43–57% after 5 d to 34–53% after 30 d in the control soils and
272 reduced from 41–55% after 5 d to 36–52% after 30 d in the 100 $mg_{oil\text{-}C}$ kg^{-1} soils.
273 Significant reduction ($P < 0.05$) was observed at higher diesel concentrations; the ^{14}C-
274 biomass reduced from 39–51% after 5 d to 22–41% after 30 d in all the 1000 $mg_{oil\text{-}C}$
275 kg^{-1} soils and from 39–56% after 5 d to 23–46% after 30 d in the 5000 $mg_{oil\text{-}C}$ kg^{-1} soils.
276 Further, the proportion of the ^{14}C-biomass formed at 5 d that was later mineralised to
277 $^{14}CO_2$ after 30 d increased from 8–20% in the control soils to 22–45% in the 1000
278 $mg_{oil\text{-}C}$ kg^{-1} soils and 17–41% in the 5000 $mg_{oil\text{-}C}$ kg^{-1} soils.
279 The amounts of non-biomass ^{14}C-residues adsorbed to clay materials (Gregorich et al.,
280 1991) were estimated by the difference between the soil-associated residual ^{14}C and
281 ^{14}C-biomass (Figure 2). The non-biomass ^{14}C-residues apparently decreased as clay
282 content increased in soil; however, the increase in the ratio of ^{14}C-biomass–to–non-
283 biomass ^{14}C-residues showed the influence of clay in a clear form. For instance, after 5,
284 15, and 30 d incubation of the control soils, the ratio of ^{14}C-biomass–to–non-biomass
285 ^{14}C-residues were 3, 4 and 5 for the low-clay Myerscough soil; 3, 7 and 5 in the
286 medium-clay Thurnham soil; and 5, 8 and 16 in high-clay Holme soil, respectively.
287 The influence of diesel concentration on the non-biomass ^{14}C-residues was more
288 discernable than that of clay content; there was consistently lower amount of non-
289 biomass ^{14}C-residues and higher ratio of ^{14}C-biomass–to–non-biomass ^{14}C-residues as
290 diesel concentration increased in soil. For example, after 30 d incubation of the low-
291 clay Myerscough soils, the non-biomass ^{14}C-residues were 8, 5 and 4% and the ratio of
292 ^{14}C-biomass–to–non-biomass ^{14}C-residues were 5, 7 and 9 in the control, 100 and 5000

293 $mg_{oil\text{-}C}$ kg^{-1} soils, respectively. For the medium-clay Thurnham soil, non-biomass ^{14}C-
294 residues were 8, 7 and 3% and the ratio of ^{14}C-biomass–to–non-biomass ^{14}C-residues
295 were 5, 7 and 14 in the control, 100 and 5000 $mg_{oil\text{-}C}$ kg^{-1} soils, respectively. For the
296 high-clay Holme soil, non-biomass ^{14}C-residues were 3, 3 and 2% and the ratio of ^{14}C-
297 biomass–to–non-biomass ^{14}C-residues were 16, 18 and 22 in the control, 100 and 5000
298 $mg_{oil\text{-}C}$ kg^{-1} soils, respectively.
299
300 *3.4. Microbial ^{14}C-turnover time, metabolic quotient, biophysical quotient and yield*
301 *coefficient*
302 The results of the modelled ^{14}C-glucose mineralisation kinetics are available as
303 supplementary data (Table S2). The appropriateness of the model was indicated by the
304 strong $r^2 \geq 0.996$ ($P < 0.0001$). Specific rate constants in the rapidly mineralising
305 fraction (K_r: 0.067–0.338 h^{-1}) were up to two orders of magnitude faster than in the
306 slowly mineralising fraction (K_s: 0.0032–0.0059 h^{-1}). Very similar values have been
307 reported for radiolabelled glucose and plant residues by other investigators (Van Veen
308 et al., 1987; Ladd et al., 1995). Turnover of ^{14}C-glucose was estimated using two
309 independent approaches: (i) microbial turnover time (MTT) was 21–33 d after 5 d
310 incubation ($MTT_{5\text{-}d}$) and 25–94 d after 30 d incubation ($MTT_{30\text{-}d}$); (ii) microbial
311 residence time (MRT) ranged from 17 to 99 d (Table 2). Both $MTT_{30\text{-}d}$ and MRT
312 correlated significantly ($r^2 = 0.714$, $n = 20$, $P < 0.0001$; Figure 3) and tended to
313 increase with increases in soil clay content and decrease with increases in diesel
314 concentration, this being significant ($P < 0.05$) in the 1000–5000 $mg_{oil\text{-}C}$ kg^{-1} soils.
315 In this study, the metabolic quotient ($q^{14}CO_2$) was used as a measure of the unit of
316 energy expended for respiratory maintenance per unit of carbon incorporated into
317 biomass for growth (i.e. metabolic activity efficiency) during the decomposition of ^{14}C-

318 glucose. Generally, the $q^{14}CO_2$ decreased as clay content increased, and increased as
319 diesel concentration increased in soils as well as decreased as incubation time increased
320 (Figure 4). There was a strong negative correlation ($r^2 > 0.920$; $P < 0.05$) between
321 $q^{14}CO_2$ and clay content of soils (Figure S1A). For example, the $q^{14}CO_2$ decreased from
322 1.70, 1.14 and 0.42 after 5 d to 0.40, 0.39 and 0.16 after 15 and to 0.20, 0.16 and 0.08
323 after 30 d in the low-clay Nether-Kellet, medium-clay Thurnham and high-clay Holme
324 soils, respectively. In particular, the effect of diesel concentration was significant ($P <$
325 0.05) in the 1000–5000 $mg_{oil\text{-}C}$ kg^{-1} soils. In a comparison between the control soil and
326 its 5000 $mg_{oil\text{-}C}$ kg^{-1} soil, $q^{14}CO_2$ increased from 1.20 to 2.29, 1.69 to 2.27, 0.42 to 0.87,
327 and from 1.14 to 1.85 for Myerscough, Nether-Kellet, Holme and Thurnham soils,
328 respectively.
329 The biophysical quotient (BQ), used as a measure of the stabilisation effect of clay,
330 significantly differed ($P < 0.001$) between soil types; the BQ was lowest in the high-
331 clay Holme soil and highest in the low-clay Nether-Kellet soil (Figure 5). This was
332 particularly evident after 15 d of incubation with strong (negatively correlated)
333 relationship ($r^2 > 0.920$; $P < 0.05$) between BQ and clay content of soils (Figure S1B).
334 For all the soils, the BQ values increased with increasing diesel concentration and
335 incubation time (Figure 5); though the values for most of the 1000 $mg_{oil\text{-}C}$ kg^{-1} soils
336 were higher than for the respective 5000 $mg_{oil\text{-}C}$ kg^{-1} soils. The BQ values slightly
337 increased during incubation from 0.47–1.02 at 5 d to 0.59–1.52 after 30 d in all control
338 soils, from 0.47–1.07 at 5 d to 0.61–1.52 after 30 d in all 100 $mg_{oil\text{-}C}$ kg^{-1} soils, and
339 significantly increased ($P < 0.001$) from 0.50–1.20 at 5 d to 0.84–2.78 at 30 d in the
340 5000 $mg_{oil\text{-}C}$ kg^{-1} soils, respectively.
341 The clay and diesel concentration affected the microbial yield coefficient (*Yc*) in the
342 soils (Figure 6). The *Yc*, used as an indicator of the utilisation efficiency, generally

343 increased as clay content increased, and decreased as diesel concentration and
344 incubation increased in soils. The correlation between *Yc* and clay content is shown
345 graphically in Figure S1C. After 5 d incubation, the *Yc* was 0.50 for the low-clay
346 Nether-Kellet soil and increased to 0.61 for the medium-clay Thurnham soil and
347 further to 0.69 for the high-clay Holme soil. In term of diesel concentration, the *Yc*
348 marginally decreased from 0.50–0.69 at 5 d to 0.40–0.63 after 30 d in all control soils,
349 decreased from 0.47–0.68 at 5 d to 0.40–0.62 after 30 d in all 100 $mg_{oil\text{-}C}$ kg^{-1} soils, and
350 further decreased from 0.41–0.67 at 5 d to 0.27–0.54 after 30 d in the 5000 $mg_{oil\text{-}C}$ kg^{-1}
351 soils.
352
353 **4. Discussion**
354 The large variations in the textural and organic carbon contents, as well as in the
355 microbial biomass sizes in the control soils notwithstanding, the results of the qCO_2
356 and RQ indicate that there were no marked differences in the metabolic state of the
357 indigenous microbiota, and in the quality of the readily available carbons in the soils.
358 The trend in the qCO_2, however, suggests that the efficiency of metabolic activity
359 decreased as clay content increased in the soils. Similar observations have been
360 reported in 16 soils obtained from major mineralogical classes of New Zealand
361 pastures (Saggar et al., 1999) and in 10 different soils under different crop
362 managements from Italy contaminated with crude oil (Franco et al., 2004). The qCO_2
363 has been used to describe the metabolic state of soil microflora (Wardle and Ghani,
364 1995; van Beelen and Doelman, 1997) and their efficiency in metabolising organic
365 carbon substrates (Dilly and Munch, 1998; Mamilov and Dilly, 2011). The RQ has
366 been used to indicate the quality of available organic carbon substrate and O_2 demand
367 for its oxidation in soil (Dilly et al., 2011).

368 The extensive mineralisation of ^{14}C-glucose during the first 3 d of incubation
369 (accounted for *ca.* 61–80% of the total extents of $^{14}CO_2$ respired after 35 d) is
370 consistent with previous studies (Bardgett and Saggar, 1994; Saggar et al., 1994;
371 Nguyen and Guckert, 2001). The decrease in the extent of mineralisation as clay
372 contents of soil increased is consistent with the findings of other studies (Saggar et al.,
373 1996; Saggar et al., 1999) and the increase in the extent of mineralisation as diesel
374 concentration increased in soil is also in agreement with other comparable studies
375 (Harden et al., 1993; Bardgett and Saggar, 1994). For example, soils treated with the
376 pesticides isoproturon, simazine, dinoterb and chloroform recorded increased CO_2
377 respiration during 0–10 d incubation (Harden et al., 1993).
378 Overall, the greater partitioning of ^{14}C-glucose to^{14}C-biomass as well as the increase in
379 the ratio of ^{14}C-biomass–to–non-biomass ^{14}C-residues as clay content of soil increased
380 further support the claims that clay plays an important role in the initial mineralisation
381 and the later decomposition of labile carbon substrate through stabilisation and
382 protection of the microorganisms (Sorensen, 1975; Ladd et al., 1995; Saggar et al.,
383 1999). The stabilisation of radiolabelled amino acid carbon formed during the short-
384 term (30 d) decomposition of ^{14}C-cellulose increased as silt + clay fraction increased in
385 seven different soils (Sorensen, 1975). In a study of 10 soils with the same type of clay
386 and under the same management but having different clay contents ^{14}C-glucose
387 incorporated as ^{14}C-biomass was greater in soils with more clay after 90 d incubation
388 (Gregorich et al., 1991). The authors also observed that the ratio of ^{14}C-biomass–to–
389 non-biomass ^{14}C-residues was consistently higher in soils with more clay and attributed
390 this to the adsorption of the non-biomass ^{14}C-residues by clay and to product utilisation
391 by a secondary population.

392 Similar to the effects of diesel observed in this present study, the effects on ^{14}C-glucose
393 mineralisation and its incorporation as ^{14}C-biomass were significant as the
394 concentration of chromium, copper and arsenic increased in a pasture soil (Bardgett
395 and Saggar, 1994). It was reported that throughout the 28 d incubation following the
396 addition of ^{14}C-glucose, the $^{14}CO_2$ respired was higher while ^{14}C-biomass formed was
397 consistently lower in the metal-contaminated soils than in the uncontaminated control
398 soil (Bardgett and Saggar, 1994). Although diesel concentration appeared not have
399 effects on the initial mineralisation of ^{14}C-glucose (up to 5 d), the later decomposition
400 of ^{14}C-carbon from either the dead ^{14}C-biomass or non-biomass ^{14}C-metabolites was
401 significant in all soils. For instance, between 5 and 30 d of incubation, about 28–68%
402 and 63–77% of non-biomass ^{14}C-residues were mineralised to $^{14}CO_2$ in the control and
403 5000 $mg_{oil\text{-}C}$ kg^{-1} soils, respectively. This indicates that this ^{14}C-pool served as both
404 sink and source of energy and nutrients, and that its availability to soil microorganisms
405 was greater in the contaminated soil.
406 The values of $MTT_{30\text{-}d}$ (25–94 d) reported in this study are comparable with the range
407 (21–75 d) given in Cheng (2009) but higher than the 29–30 d reported by
408 Blagodatskaya et al. (2011). The values of MRT (17–99 d) are also comparable with
409 the range (42–134 d) reported in Saggar et al. (1999). The failure to observe any
410 influence of clay on ^{14}C-turnover during the early stage of incubation ($MTT_{5\text{-}d}$) can be
411 attributed to the requirement for steady-state conditions to correctly estimate MTT
412 (Blagodatskaya et al., 2011). Results for both $MTT_{30\text{-}d}$ and MRT indicate that ^{14}C-
413 turnover was significantly ($P < 0.05$) influenced by clay content; ^{14}C-turnover was
414 approximately two times faster in the other soils than in the high-clay Holme soil. The
415 results are in agreement with those of other investigators (Ladd et al., 1995; Saggar et
416 al., 1999). Diesel, especially at higher concentrations (1000–5000 $mg_{oil\text{-}C}$ kg^{-1}) further

417 increased the ^{14}C-turnover rates in the soils. This is attributable, in part, to changes in
418 the microbial community structure and, in part, to the influence on clay stabilisation
419 capacity as a result of larger amounts of the hydrophobic organic contaminants being
420 adsorbed to sites on clay materials in soil. Hydrophobic organic contaminants are
421 believed to strongly adsorb onto soil colloids, such as clay and humic substances
422 (Stokes et al., 2005).
423 Linked parameters, such as the $q^{14}CO_2$, BQ and *Yc*, are thought to be more sensitive to
424 the effect of perturbations than data from respiration rates or amounts of biomass alone
425 (Wardle and Ghani, 1995; van Beelen and Doelman, 1997). Collectively, the results of
426 these parameters and their strong correlations with clay content indicate that clay
427 influences the metabolic activity of soil microorganisms and their efficiency to utilise
428 labile carbon substrate. The increase in the $q^{14}CO_2$ and BQ values along the diesel
429 concentration gradient suggests a perturbation effect while the decrease over time
430 indicates the abatement of such effect (Wardle and Ghani, 1995). Further, the results
431 showed that the existing microorganisms were more active in the amended soils, and
432 that their metabolic activity increased significantly at higher diesel concentrations
433 (1000–5000 $mg_{oil\text{-}C}\ kg^{-1}$) in soil. The decrease in the *Yc* values as diesel concentration
434 as well as incubation time increased indicates the increased demand for labile carbon
435 substrate by the actively-growing hydrocarbon-degrading microorganisms for cell
436 maintenance, not resulting in additional biomass formation. This finding provides
437 insight into the role of labile carbon substrates in the biodegradation of petroleum
438 hydrocarbons in soil.
439 The findings of this current study align with that of Bardgett and Saggar (1994) who
440 observed that the rate of $^{14}CO_2$ respiration was faster in heavy metal contaminated soils
441 than in the uncontaminated control, resulting in both (total respired C):(total biomass-

C) and (respired $^{14}CO_2$):(^{14}C-biomass) being greater in the contaminated soils. Thus, similar to the effect of increasing concentration of heavy metals, it is suggested that the indigenous microorganisms in diesel-contaminated soil expend more energy for maintenance requirements, and are less efficient in their utilisation of labile substrates for biomass synthesis. To the authors' knowledge, this is the first report of the combined influence of petroleum hydrocarbon contaminants and soil texture on the microbial utilisation efficiency and short-term turnover of labile carbon substrate in soil.

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630 **Tables**

631 Table 1: Selected physicochemical and microbiological properties of soils.

Soil Characteristics	Myerscough	Nether-Kellet	Holme	Thurnham
Classification	Typical Stagnogley	Typical brown-earth	Earthy oligo-fibrous peat	Typical humic alluvial gley
Grid reference	SD 496402	SD 491655	SD 511775	SD 447543
Texture	Sandy loam	Loam	Silty clay	Clay loam
Particle size analysis (g kg^{-1})				
Sand	556.3	553.9	104.5	387.7
Silt	249.6	267.7	473.7	344.4
Clay	194.1	178.4	421.8	277.9
pH (dH_2O)	6.53	5.44	7.50	6.93
Soil organic matter (%; LOI)	4.82	9.33	27.15	10.25
Total organic carbon, $C_{org.}$ (g kg^{-1})	17.0	29.9	195	34.8
Total nitrogen (g kg^{-1})	1.4	2.5	13.2	2.6
Biomass-C (mg kg^{-1})	1072	1362	2344	2040
Fungal/Bacterial Biomass-C ratio	1.31	1.09	1.02	1.25
Bacterial counts (x 10^3 CFU g^{-1})	363	49	2590	307
qCO_2 (µg CO_2-C mg^{-1} biomass-C h^{-1})	5.02	5.22	3.86	4.72
RQ (µg CO_2 μg^{-1} O_2)	1.10	1.00	1.23	1.25
∑16 USEPA PAHs (µg kg^{-1})	30	103	92	42
Soil description[a]	Low–Low	Low–Medium	High–High	Medium–Medium

632 qCO_2: Metabolic quotient; RQ: Respiratory quotient; [a] relative clay and SOM contents of soils.
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634

635 Table 2: Effect of diesel concentration on microbial ^{14}C-turnover time in soils.

Soil type	Concentration (mg kg^{-1})	$MTT_{5\text{-}d}$ (d)	$MTT_{30\text{-}d}$ (d)	MRT (d)[¶]
Myerscough	0	33	41	53
	10	32	40	56
	100	28	34	42
	1000	23	33	17
	5000	23	25	24
Nether-Kellet	0	26	41	56
	10	30	49	68
	100	25	41	59
	1000	27	26	23
	5000	21	30	26
Holme	0	33	94	99
	10	32	69	93
	100	28	61	49
	1000	23	28	28
	5000	23	26	32
Thurnham	0	25	59	46
	10	24	53	42
	100	25	42	44
	1000	22	29	39
	5000	21	26	38

636 [¶] Calculated using the biexponential Equation 6

637 **Figures**

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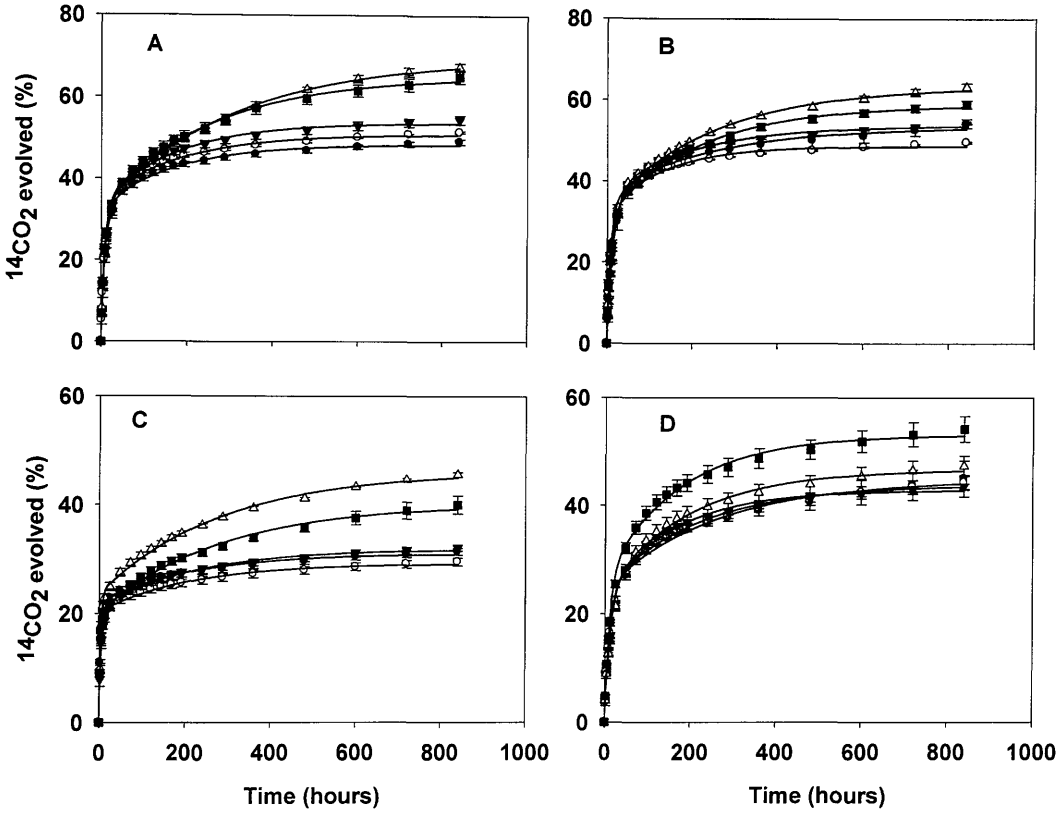

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642 Figure 1: Effect of diesel concentration on ^{14}C-glucose mineralisation in (A)
643 Myerscough, (B) Nether-Kellet, (C) Holme and (D) Thurnham soils amended with oil
644 at 0 (●), 10 (○), 100 (▼), 1000 (Δ) and 5000 $mg_{oil\text{-}C}\ kg^{-1}_{soil}$ (■). Symbols represent the
645 mean of three replicates; errors bars indicate one standard error of mean (SEM) and
646 were not visible when smaller than the symbol for the mean. Smooth lines represent the
647 best fit of the 2-compartment first-order model (equation 1).

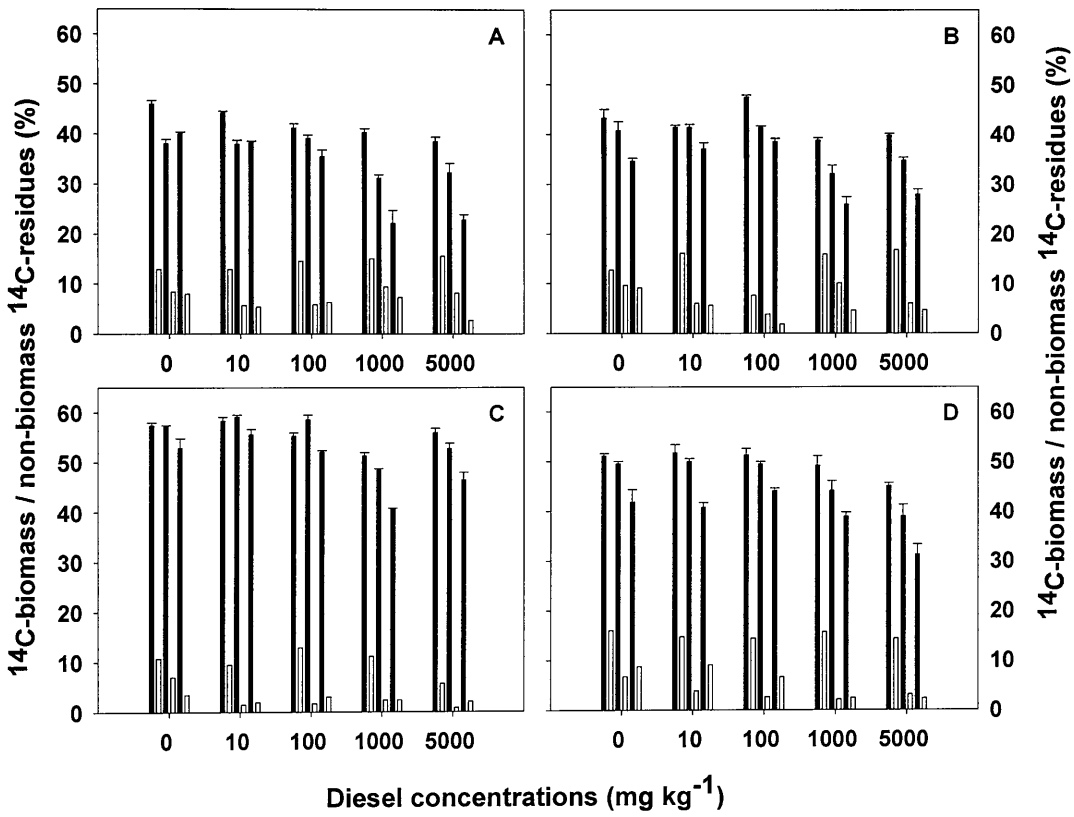

Figure 2: Effect of diesel concentration on microbial uptake of ^{14}C-glucose as ^{14}C-biomass (■) and non-biomass ^{14}C-products (□) in (A) Myerscough, (B) Nether-Kellet, (C) Holme and (D) Thurnham soils.

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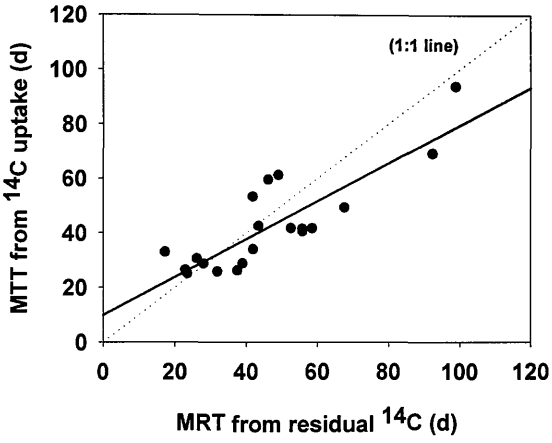

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658 Figure 3: A comparison of microbial ^{14}C-turnover times calculated from the residual

659 ^{14}C (MRT) and microbial ^{14}C-uptake (MTT) data (MTT = 9.85 + 0.70MRT; $r = 0.845$,

660 n = 20, $P < 0.0001$).

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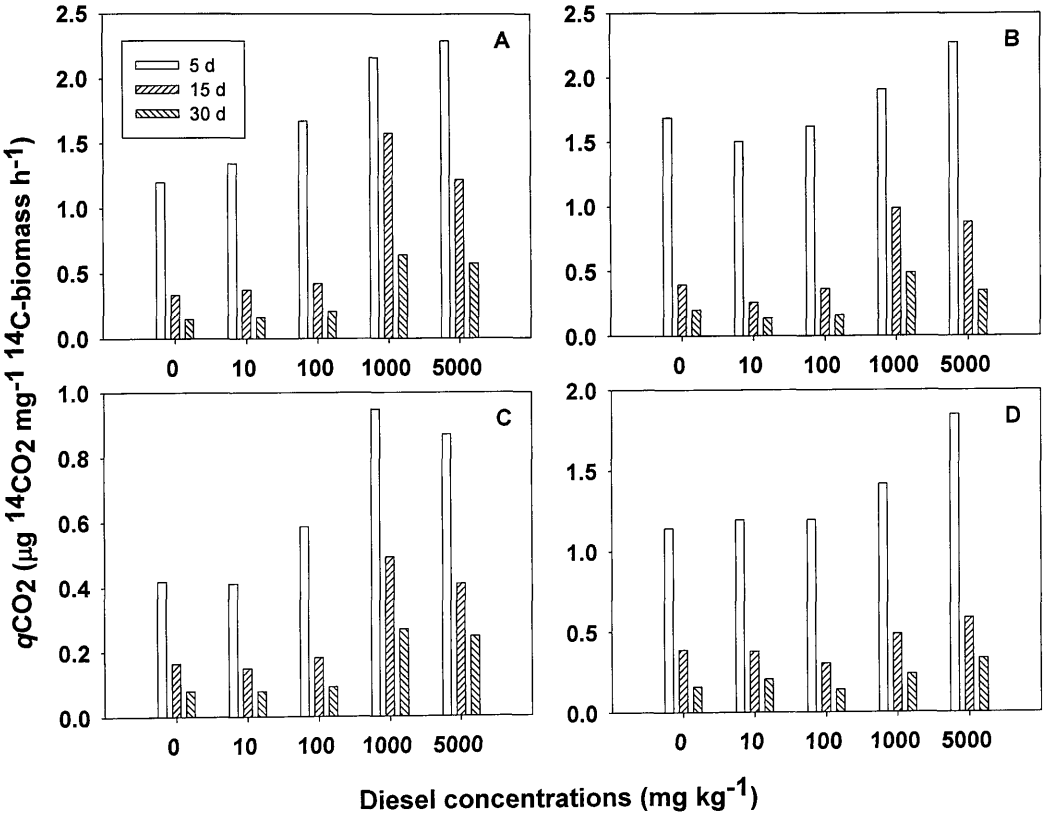

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665 Figure 4: Effect of diesel concentration on metabolic activity ($q^{14}CO_2$) in (A)
666 Myerscough, (B) Nether-Kellet, (C) Holme and (D) Thurnham soils after incubation
667 for 5, 15, and 30 d.

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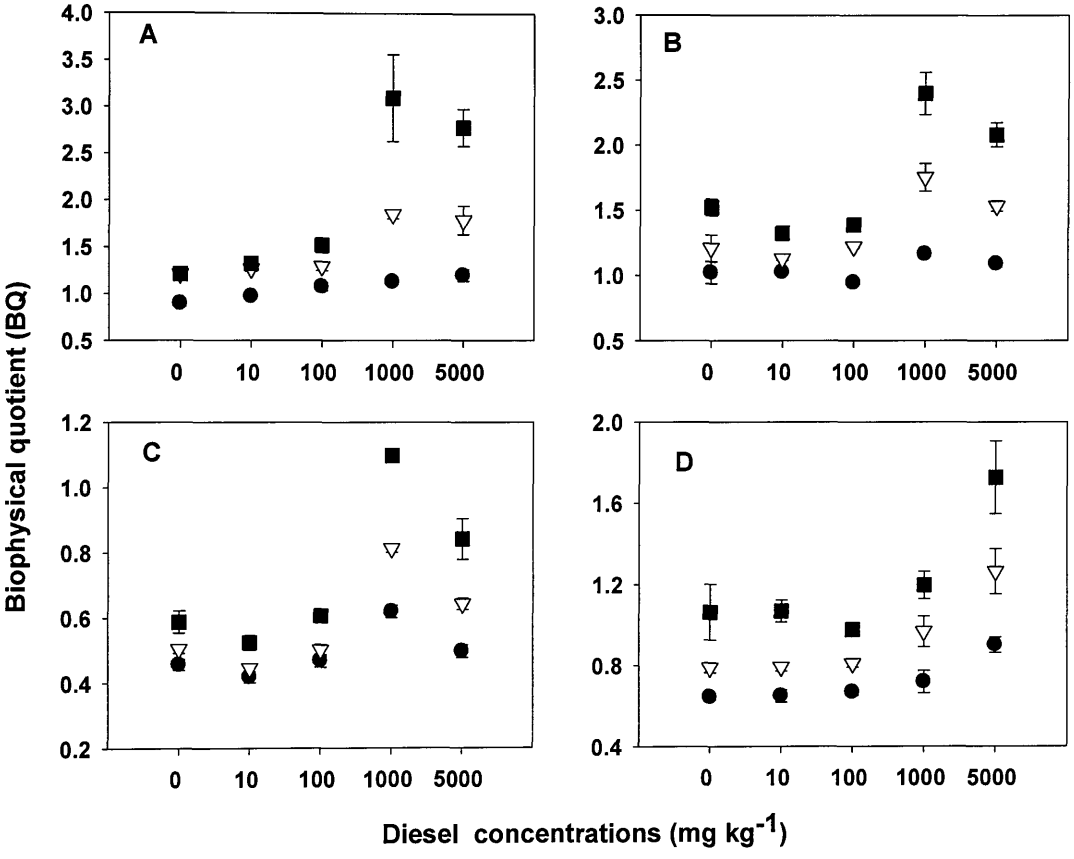

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672 Figure 5: Effect of diesel concentration on biophysical quotient (BQ) in (A)
673 Myerscough, (B) Nether-Kellet, (C) Holme and (D) Thurnham soils after incubation
674 for 5 (●), 15 (▽), and 30 d (■).

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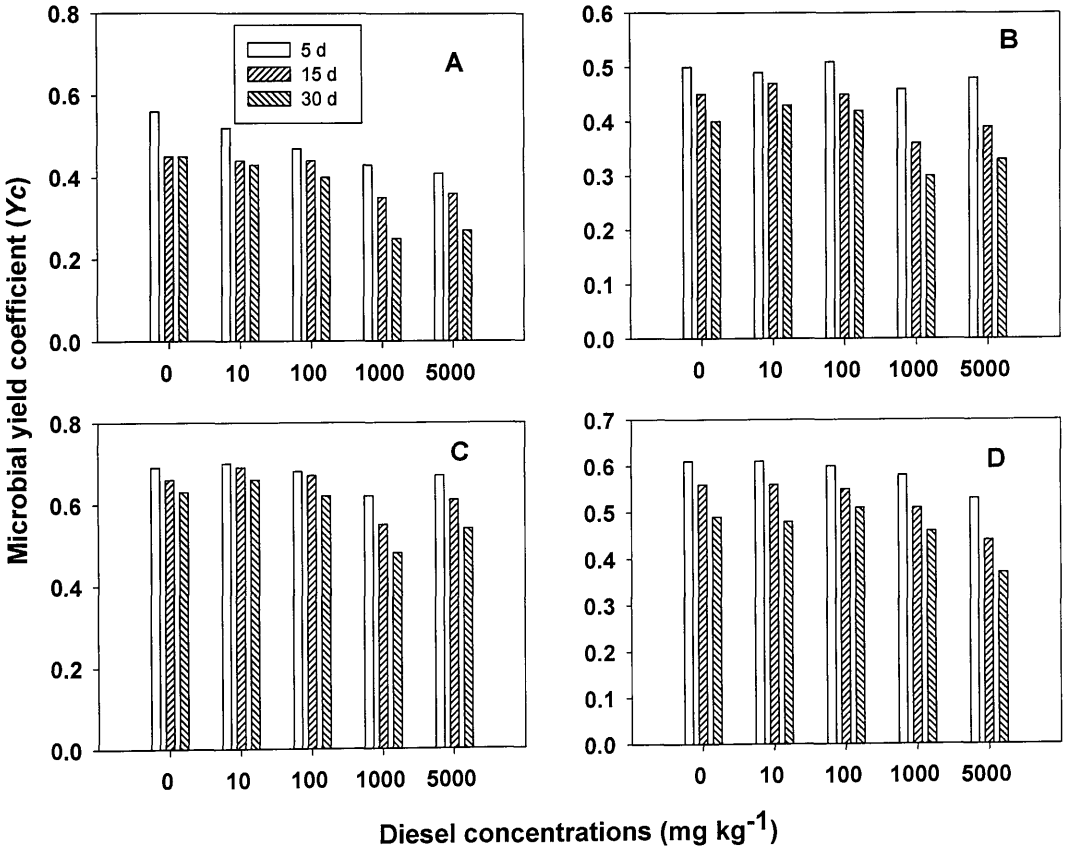

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679 Figure 6: Effect of diesel concentration on microbial yield coefficient (*Yc*) in (A)

680 Myerscough, (B) Nether-Kellet, (C) Holme and (D) Thurnham soils after incubation

681 for 5, 15, and 30 d.

Supplementary materials

Supplementary Table S1: Effect of diesel on microbial mineralisation presented as lag time (hours), percent maximum rate (% h^{-1}), percent cumulative $^{14}CO_2$ respired (%) in (A) Myerscough, (B) Nether-Kellet, (C) Holme and (D) Thurnham soils.

Soil (d)	Concentration (mg-C kg^{-1})	Lag time (h)	Maximum rate (% h^{-1})	T_{max} (h)	Overall extent (%)
A	0	$1.52 \pm 0.12^{\S aA\dagger}$	3.33 ± 0.25^{abA}	2	48.50 ± 0.63^{aAB}
	10	1.96 ± 0.34^{aAC}	3.25 ± 0.07^{aA}	4	50.85 ± 0.24^{abA}
	100	1.51 ± 0.18^{aA}	3.60 ± 0.24^{abA}	4	53.76 ± 1.01^{bA}
	1000	1.21 ± 0.03^{aA}	4.15 ± 0.10^{bA}	2	66.14 ± 1.11^{cA}
	5000	1.47 ± 0.09^{aA}	3.76 ± 0.10^{abA}	4	63.06 ± 1.56^{cA}
B	0	1.67 ± 0.00^{aA}	3.00 ± 0.01^{aA}	2	52.62 ± 1.39^{aA}
	10	1.52 ± 0.07^{abBC}	3.30 ± 0.15^{aA}	2	49.07 ± 0.22^{bA}
	100	1.79 ± 0.20^{aAC}	2.86 ± 0.28^{aA}	2	53.49 ± 0.09^{aA}
	1000	1.06 ± 0.05^{bA}	4.73 ± 0.20^{bA}	2	61.95 ± 0.86^{dA}
	5000	1.37 ± 0.16^{abA}	3.76 ± 0.51^{abA}	2	58.07 ± 0.90^{cAB}
C	0	0.91 ± 0.04^{aC}	5.50 ± 0.26^{aB}	2	30.92 ± 0.63^{aB}
	10	1.07 ± 0.04^{aB}	4.67 ± 0.15^{aB}	2	29.10 ± 0.69^{aC}
	100	1.35 ± 0.26^{aA}	3.94 ± 0.63^{aA}	2	31.60 ± 0.62^{aC}
	1000	1.03 ± 0.11^{aA}	4.93 ± 0.48^{aA}	2	44.57 ± 0.26^{cB}
	5000	1.12 ± 0.07^{aA}	4.52 ± 0.32^{aA}	2	38.92 ± 1.54^{bC}
D	0	2.29 ± 0.23^{aB}	2.69 ± 0.12^{aA}	4	43.81 ± 2.81^{aB}
	10	2.55 ± 0.18^{aA}	2.83 ± 0.09^{aA}	4	43.42 ± 1.27^{aB}
	100	2.51 ± 0.15^{aBC}	2.46 ± 0.21^{aA}	4	43.00 ± 0.52^{aB}
	1000	2.37 ± 0.15^{aB}	2.44 ± 0.23^{aB}	4	46.48 ± 1.86^{bB}
	5000	2.06 ± 0.10^{aB}	2.96 ± 0.19^{aA}	4	53.17 ± 2.22^{cB}

[§] Where appropriate, values are means of three replicates ± standard errors

[†] Different lower-case letters down the column for each "Soil type" indicate means that are statistically different ($P < 0.05$). Different upper-case letters down the column for corresponding "Concentration" indicate means that are statistically different ($P < 0.05$).

691 Supplementary Table S2: Effect of diesel on the kinetic parameters of model fit to ^{14}C-

692 glucose mineralisation data in soils.[§]

Soil type	Concentration ($mg_{oil\text{-}C}\ kg^{-1}$)	A (%)	F_r	K_r (h^{-1})	B (%)	F_s	K_s ($10^{-3}\ h^{-1}$)	r^2_{adj}
Myerscough	0	32.65 (0.76)	0.68	0.106 (0.004)	15.71 (0.69)	0.32	5.90 (0.60)	0.999
	10	34.53 (0.80)	0.68	0.095 (0.004)	16.34 (0.71)	0.32	5.50 (0.50)	0.999
	100	33.51 (0.82)	0.62	0.125 (0.006)	20.18 (0.75)	0.38	5.80 (0.50)	0.998
	1000	33.01 (0.90)	0.47	0.125 (0.007)	36.64 (0.90)	0.53	3.20 (0.30)	0.998
	5000	33.53 (0.76)	0.51	0.121 (0.006)	31.61 (0.71)	0.49	3.90 (0.30)	0.998
Nether-Kellet	0	36.93 (1.51)	0.69	0.068 (0.005)	16.56 (1.24)	0.31	4.00 (0.90)	0.996
	10	32.53 (1.25)	0.67	0.102 (0.007)	16.12 (1.15)	0.33	5.30 (0.90)	0.998
	100	36.97 (1.10)	0.69	0.067 (0.003)	16.82 (0.94)	0.31	5.00 (0.70)	0.999
	1000	33.63 (1.03)	0.53	0.123 (0.008)	29.87 (0.97)	0.47	4.00 (0.40)	0.997
	5000	31.33 (0.83)	0.53	0.122 (0.007)	27.53 (0.76)	0.47	4.60 (0.40)	0.998
Holme	0	21.91 (0.29)	0.71	0.338 (0.015)	9.11 (0.33)	0.29	4.70 (0.50)	0.997
	10	20.29 (0.25)	0.69	0.309 (0.013)	8.92 (0.28)	0.31	4.70 (0.40)	0.999
	100	21.02 (0.34)	0.66	0.263 (0.013)	10.80 (0.37)	0.34	4.60 (0.40)	0.997
	1000	23.64 (0.44)	0.51	0.291 (0.018)	22.48 (0.60)	0.49	3.50 (0.30)	0.997
	5000	20.19 (0.48)	0.50	0.299 (0.024)	19.98 (0.64)	0.50	3.60 (0.30)	0.996
Thurnham	0	24.72 (1.00)	0.55	0.088 (0.007)	20.12 (0.88)	0.45	3.80 (0.50)	0.996
	10	24.74 (0.99)	0.56	0.086 (0.007)	19.11 (0.85)	0.44	4.60 (0.60)	0.997
	100	23.80 (1.25)	0.56	0.082 (0.007)	19.06 (1.12)	0.44	6.10 (0.70)	0.997
	1000	24.32 (1.33)	0.52	0.075 (0.007)	22.41 (1.15)	0.48	5.00 (0.60)	0.997
	5000	26.81 (1.41)	0.50	0.090 (0.009)	26.31 (1.26)	0.50	5.60 (0.60)	0.997

693 [§] Where appropriate, values are means of three replicates (standard errors of means).
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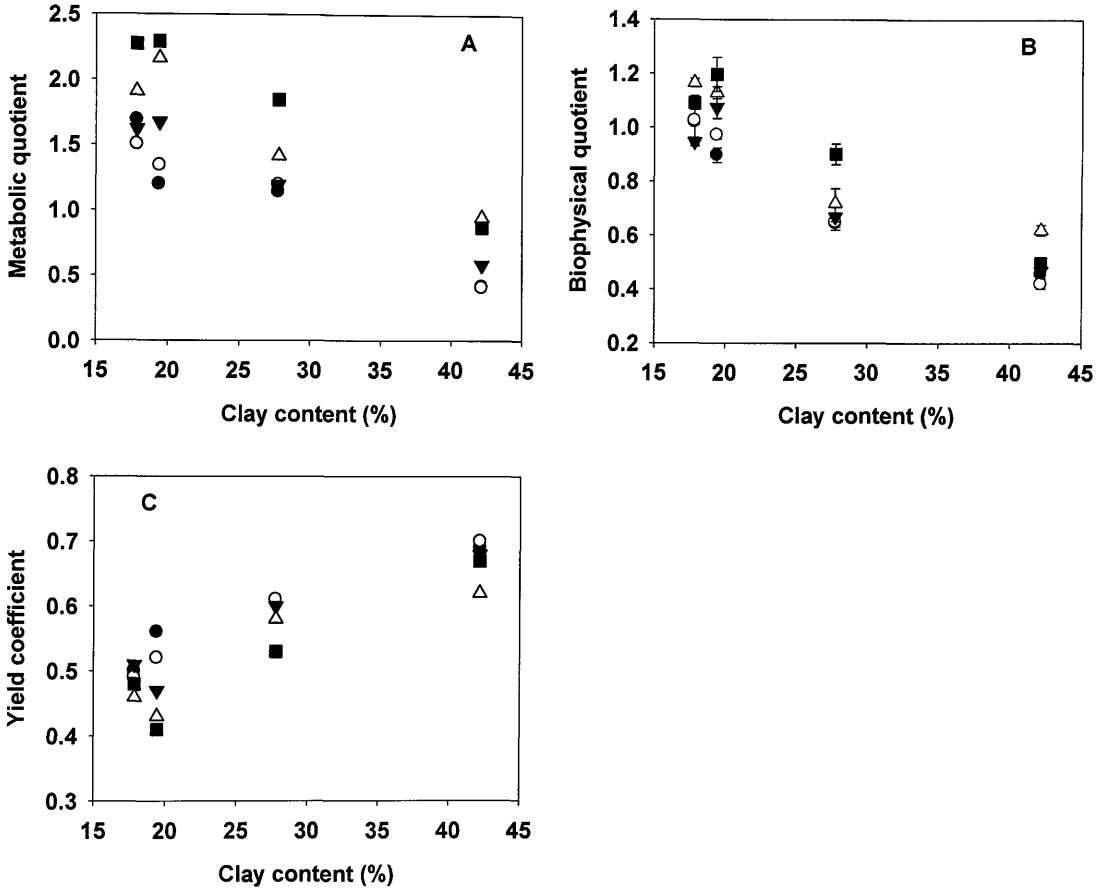
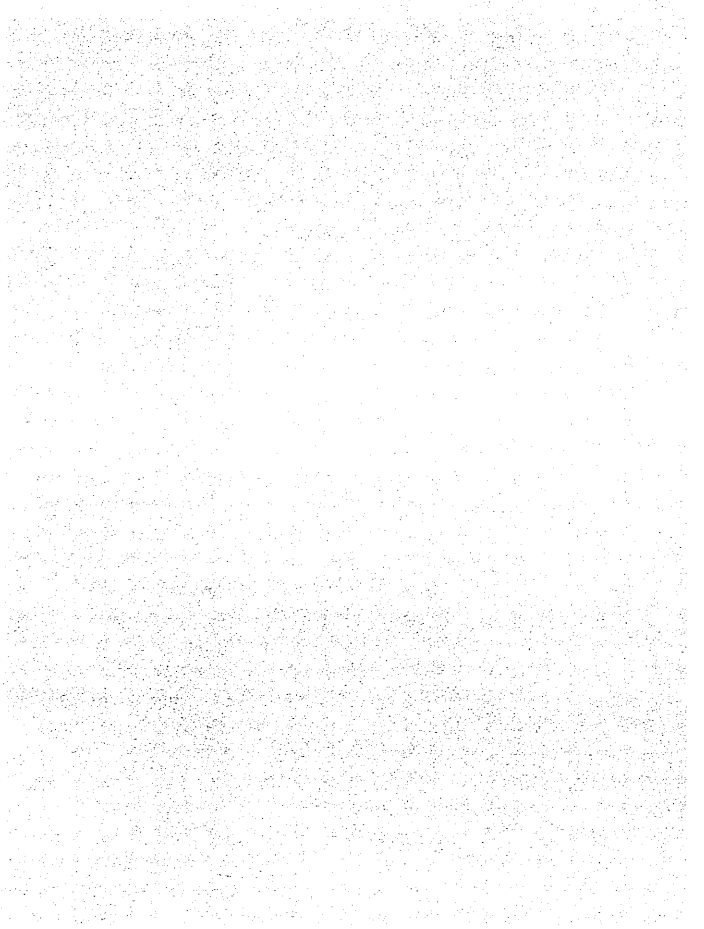


Supplementary Figure S1: Relationship between clay content and (A) metabolic quotient ($q^{14}CO_2$), (B) biophysical quotient (BQ) and (C) microbial yield coefficient (*Yc*) in the control soils (●) and soils amended with diesel to 10 (○), 100 (▼), 1000 (Δ) and 5000 $mg_{oil\text{-}C}\ kg^{-1}$ (■). The data were selected from the 5 d incubation samples.

Appendix

Paper XII



Appendix

Paper XII

The effect of rhizosphere soil and root tissue amendment on microbial mineralisation of target ^{14}C-hydrocarbons in contaminated soil

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Abstract

This study investigated the development of microbial mineralisation of ^{14}C-naphthalene, ^{14}C-phenanthrene, ^{14}C-hexadecane or ^{14}C-octacosane in freshly spiked and 28 d aged soils amended with rhizosphere soil or root tissues at 5% (wet weight). Soil amended with rhizosphere soil or root tissues of reed canary grass (*Phalaris arundinacea*), channel grass (*Vallisneria spiralis*), blackberry (*Rubus fructicosus*) and goat willow (*Salix caprea*) exhibited enhanced ($P < 0.001$) levels of ^{14}C-naphthalene and ^{14}C-phenanthrene mineralisation at 0 d. In contrast, there were no enhancement in ^{14}C-hexadecane or ^{14}C-octacosane mineralisation in freshly spiked soils amended with rhizosphere soil or root tissue. Degradation of polycyclic aromatic hydrocarbons (PAHs) was further enhanced by pre-exposure of soil to PAH, but the extents of hydrocarbon mineralisation were not enhanced ($P > 0.05$) by addition of rhizospere soil or root tissue after 28 d. This study suggest that organic chemicals in roots and/or rhizosphere can stimulate the devlopment of microbial degradative capabilities and PAH biodegradation in freshly contaminated soil.

Capsule: Adapted rhizospheric microorganisms or root tissues from contaminated site can be used as bioaugments or biostimulant during PAH bioremediation.

Keywords: *Hydrocarbons*; *Mineralisation*; *PAH*; *Rhizosphere soil*; *Root tissue*

1. Introduction

Phytoremediation involves the use of plants and their associated microbes to remove, tranform and/or degrade inorganic and organic contaminants in soil, sediments and groundwater (Hughes et al., 1996; Susarla et al., 2002; Arthur et al., 2005). Phytoremediation strategies for organic comtaminants such as petroleum hydrocarbons can be grouped into direct phytoremediation and phytoremediation *ex planta* (Anderson et al., 1993; Salt et al., 1998). The latter is based on a synergistic relationship between root exudates and metabolic activities of rhizosphere-associated microbes (Phillips et al., 2008). A number of studies has shown that plants enhance microbial degradation of hydrocarbon contaminants in soil (Aprill and Sims, 1990; Anderson et al., 1993; Gunther et al., 1996; Nichols et al., 1997; Miya and Firestone, 2001; Phillips et al., 2008; Phillips et al., 2012). Plants, in combination with microbes, play a vital role in the decontamination of polluted environments through a series of processes and metabolic transformations. Anderson et al. (1993) provided substantial evidence for the potential role of plants in hydrocarbons degradation through the rhizosphere effect; wherein plants exude organic compounds from their roots, influencing the abundance, diversity, and/or activity of rhizospheric hydrocarbon-degrading microbes.

Plant roots transfer approximately 66–243 mg C (g root)$^{-1}$ day^{-1} (Prikryl and Vancura, 1980) and nearly 5–21% of all photosynthetically fixed carbon to the rhizosphere through root exudates (Marschner, 1995; Nguyen, 2003; Nguyen, 2009). Root exudates may take several forms: low molecular weight compounds (simple sugar, amino acids, fatty acids, organic acids, phenolics, aliphatic and/or aromatic compounds) or high weight polymers, such as polysaccharides and polygalatic acids (Curl and Truelove, 1986; Marschner, 1995). They can be utilised by some soil microflora as growth

57 substrates (Vokou et al., 2002), and can act as co-metabolites for the degradation of
58 persistent organic pollutants (Donnelly et al., 1994; Haby and Crowley, 1996; Hegde
59 and Fletcher, 1996; Singer et al., 2003). It has been found that the respiration of
60 rhizosphere soil is greater than that of the bulk soil, since CO_2 can originate not only
61 from microbial respiration of soil organic C, but also from root respiration and
62 microbial decomposition of rhizodeposition (Nannipieri et al., 2008). The emission and
63 utilisation of volatile organic compounds (VOCs) within the rhizosphere form a
64 significant part of the carbon cycle (Misra et al., 1996; Misra and Pavlostathis, 1997).
65 Root exudates potentially supply microbes with micronutrients and the exudation of
66 organic compounds from roots is an important process in mediating plant–microbe
67 interactions. The emission of VOCs within soil, either by roots or by decomposing
68 biomass may enhance the biodegradation of organic contaminants (Hernandez et al.,
69 1996; Hernandez et al., 1997; Tandlich et al., 2001). In a recent study, Rhodes et al.
70 (2007) demonstrated that the addition of biogenic VOCs to soils enhanced the
71 biodegradation of organic contaminants. Aliphatic and aromatic acids occur naturally
72 in plant roots and whether or not these compounds are present in the rhizosphere in
73 quantities sufficient to stimulate mineralisation of organic contaminants, such as PAHs,
74 in soil is not fully understood. Although phytoremediation has been extensively
75 investigated, more information about specific mechanisms and the complex role of root
76 exudates during biodegradation of organic contamiants is still needed.
77 This study investigated the influence of rhizosphere soil or root tissue of reed canary
78 grass (*Phalaris arundinacea*), channel grass (*Vallisneria spiralis*), blackberry (*Rubus*
79 *fructicosus*) and goat willow (*Salix caprea*) on microbial mineralisation of target ^{14}C-
80 hydrocarbons by indigenous soil microbes.
81

2. Materials and methods

2.1 Materials

The chemicals, naphthalene (>96%), [7-^{14}C]naphthalene (specific activity 2–10 mCi mmol^{-1}, radiochemical purity >95%), phenanthrene (>96%); [9-^{14}C]phenanthrene (50 mCi mmol^{-1}, 99.6%), *n*-hexadecane (>99%), [1-^{14}C]*n*-hexadecane (7.5 mCi mmol^{-1}, 98.6%), octacosane (≤99%), [14, 15-^{14}C]octacosane (7.5 mCi mmol^{-1}, radiochemical purity 98%) were all acquired from Sigma–Aldrich, UK. Fisher Scientific UK supplied the nutrient agar powder, sodium hydroxide (NaOH), and the minimal basal salts (MBS) solution reagents. The plate count agar (PCA) was obtained from Oxoid Ltd., UK. The 250 ml Schott Duran® bottles with Teflon™ lined screw caps were supplied by Schott, UK and the metal fittings used to make the respirometers were obtained from RS, UK. The Goldstar liquid scintillation cocktail, 7 ml and 20 ml glass scintillation vials were supplied by Meridian, UK.

2.2 Sample preparation and characterisation

A pristine pasture soil was collected (A horizon; 5–20 cm) from Myerscough Agricultural College (Lancashire, UK). Plants with attached roots and root-associated rhizosphere soil used in this study were sampled from the former Shell ICI Refinery site at Middleton Woods (Lancaster, UK) which has been derelict since 1977 and has developed a range of wildlife habitats since it was decommisioned. The plants sampled included reed canary grass (*Phalaris arundinacea*), channel grass (*Vallisneria spiralis*), blackberry (*Rubus fructicosus*) and goat willow (*Salix caprea*). The field moisture content of the pasture soil was determined, in triplicate, by oven drying at 105 °C for 24 h. The pasture soil was air-dried for 24 h and then passed through a 2-mm seive to remove stones and residual plant materials. The soil that was removed from around the

roots was used as rhizosphere soil. The homogenised soil, rhizosphere soil and plant root samples were stored in the dark at 4 °C prior to the experiment.

The physicochemical properties of the pasture soil was determined using standard techniques (Table 1). Soil had a clay loam texture (determined using sedimentation, 40 g air dried soil), pH was determined using a calibrated pH meter (10 g soil:25 ml dH_2O), and organic matter content was determined using weight loss on ignition at 450 °C for 24 h. A Carlo Erba CHNS–OEA 1108 CN–Elemental analyzer was used to determine the total carbon and nitrogen contents. The phosphate content of the sample was determined by acid digestion with HNO_3. Phosphate reducing agent (neutralised with NaOH) was used to develop the characteristic blue colour for spectrometric determination at 882 mm (Cecil CE 1011 UV Spectrometer).

2.3 Mineralisation of target ^{14}C–hydrocarbons in soil

The air-dried homogenised pasture soil was rehydrated to its original field moisture content of 35% by weight and spiked with a 10 mg kg^{-1} concentration of naphthalene, phenanthrene, hexadecane or octacosane after the method described by Doick et. al (2003). The carrier solvent in the soils was allowed to vent for 2 h under a fumehood to minimise the impact on the indigenous soil microflora. Homogeneity of the spiked soil was achieved by blending, wherein soils were manually mixed in glass bowls using a stainless steel spoon (Doick et al., 2003). Each prepared condition was placed in amber glass microcosms in triplicates with loosely fitted Teflon–lined screw caps to allow ambient oxygen exchange. All microcosoms were stored in the dark at 21 ± 1 °C, relative humidity of 45%, and sampled at 0 and 28 d for both respirometric assays and microbial analysis.

Microbial mineralisation of ^{14}C-naphthalene, ^{14}C-phenanthrene, ^{14}C-hexadecane or

132 ^{14}C-octacosane was determined using respirometric techniques following the procedure
133 developed by Reid et al. (2001). Respirometers were set up in triplicates using
134 modified 250 ml Schott Duran® bottles containing 10 g ± 0.1 g soil (wet weight) and
135 30 ml sterile minimal basal solution (Carmichael and Pfaender, 1997; Doick and
136 Semple, 2003). The respirometers were spiked with the respective hydrocarbon
137 standards prepared in toluene to deliver ^{12}C-hydrocarbon concentration of 10 mg kg^{-1}
138 soil dry wt. with an associated ^{14}C-activity of 83 Bq g^{-1} soil dry wt. Different sets of
139 respirometers were amended with rhizosphere soil or root tissues at 5% (wet weight
140 basis). Control respirometers containing only rehydrated pasture soil were set up as
141 analytical blanks. Glass vials (7 ml) containing 1 ml of 1 M NaOH were attached to the
142 Teflon-lined lids to trap $^{14}CO_2$ that had evolved during the mineralisation assay. The
143 sealed respirometers were incubated at 21 ± 1 °C and shaken at 100 rpm on a benchtop
144 orbital shaker (Janke and Kunkel, IKA®–Labortechnik KS 250). The spent $^{14}CO_2$ traps
145 were sampled every 24 h over 14 d period and 5 ml of Goldstar scintillation fluid added
146 to the spent trap. Samples were stored in the dark for >12 h before the level of ^{14}C-
147 activity was quantified by liquid scintillation counting (Packard Canberra Tri-Carb®
148 2300TR). The rate and extent of hydrocarbon mineralisation in the soil slurry was
149 calculated based on the percentage of trapped $^{14}CO_2$ over the total pool of ^{14}C-labelled
150 carbon.
151
152 *2.4 Enumeration of cell numbers of total heterotrophic bacteria and hydrocarbon-*
153 *degrading microflora*
154 The enumeration of total heterotrophic bacteria (THB) and indigenous hydrocarbon-
155 degrading microflora was carried out following standard plate count techniques (Lorch
156 et al., 1995; Foght and Aislabie, 2005). In brief, 1 ± 0.1 g soil was extracted with 10 ml

157 quarter-strength sterile Ringer's solution and 0.1 ml extracts were then serially diluted
158 using aseptic technique. Serial dilutions of suspension of bacteria (0.01 ml) were
159 inoculated onto plate count agar for THB and agar-agar plates amended with
160 naphthalene, phenanthrene, *n*–hexadecane, or *n*–octacosane as the sole C-source for the
161 respective hydrocarbon degraders. The inoculated plates were incubated at 25 ± 0.5 °C
162 and the microbial cell numbers of THB was counted after 48 h and 72 h for
163 hydrocarbon degraders. The microbial cell numbers are expressed as colony forming
164 units per gram soil dry weight (CFUs g^{-1}).
165
166 *2.5 Statistical analysis*
167 The results were analysed at each time point and statistically verified using *t*–tests after
168 normality and equal variance tests were conducted (SigmaStat®, Version 3.5, Systat
169 Software Inc., Tukey test, $P \leq 0.05$). The mineralisation profiles of the different
170 treatment conditions are presented using SigmaPlot® (Version 12.2, Systat Software
171 Inc.).
172
173 **3. Results**
174 *3.1 Mineralisation of ^{14}C-naphthalene in soils amended with rhizosphere soil or root*
175 *tissues*
176 Mineralisation of ^{14}C-naphthalene was monitored in freshly spiked and 28 d aged soils
177 amended with rhizosphere soil or root tissues using respirometric assays (Figure 1;
178 Tables 2–3). The indigenous soil microflora in contaminated soils amended with
179 rhizosphere soil or root tissues rapidly mineralised naphthalene as indicated by the
180 relatively short lag phases (Tables 2–3). The lag phases prior to extensive
181 mineralisation of ^{14}C–naphthalene (≥ 5% added ^{14}C-activity) ranged from 3.27±0.18

182 (i.e. reed canary grass root tissue) to 72.20±0.04 h (i.e. unamanded soil) at 0 d, and
183 from 3.42±0.10 (i.e. reed canary grass root tissue) to 4.15±0.06 h (i.e. chanel grass root
184 tissue) after 28 d. The lag phases in soils amended with root tissues were significantly
185 ($P < 0.001$) shorter compared to soils amended with rhizosphere soil and unamended
186 soil. After 28 d soil–contaminant contact time, there were subtle differences in the lag
187 phases between various treatment conditions. Soils amended with rhizosphere soil
188 exhibited the shortest ($P < 0.001$) lag phases relative to other treatments. The rates of
189 ^{14}C-naphthalene mineralisation were significantly ($P < 0.05$) faster in soils amended
190 with rhizosphere soil and root tissues compared to the unamended soil at 0 d (Table 2).
191 After 28 d ageing period, there were no significant ($P > 0.05$) differences in the
192 maximum rates of ^{14}C-naphthalene mineralisation in soil amended with rhizosphere
193 soil or root tissues and the unamended soil (Table 3).
194 The extents of ^{14}C-naphthalene mineralisation ranged from 42.79±2.32 to 56.28±1.75%
195 in soils amended with rhizosphere soil; from 51.82±1.72 to 59.24±1.55% in soils
196 amended with root tissues; and 36.31±1.51% in the unamended soil at 0 d (Figure 1;
197 Table 2). Enhanced extents of ^{14}C-naphthalene mineralisation were observed in soils
198 amended with rhizosphere soil or root tissues ($P < 0.001$) in relative to unamended soil.
199 The highest extents of ^{14}C-naphthalene mineralisation were observed in soils amended
200 with root tissues of reed canary grass (59.24±1.55%) and goat willow (58.69±0.16%),
201 while the lowest mineralisation extent was observed in soil amended with chanel grass
202 rhizosphere soil (42.79±2.32%). With the exception of soil amended with chanel grass,
203 there were no significant ($P > 0.05$) differences in the extents of ^{14}C-naphthalene
204 mineralisation between rhizopshere soil and root tissues amended soils. After 28 d
205 soil–contaminat contact time, mineralisation extents ranged from 47.96±1.49 to
206 52.47±1.72% in soils amended with rhizosphere soil; from 48.96±1.18 to 52.31±0.84%

207 in soils amended with root tissues; and 46.39±1.77% in the unamended soil (Figure 1;
208 Table 3). There were no significant ($P > 0.05$) differences in the extents of ^{14}C-
209 naphthalene mineralisation between various amended soil treatments and the
210 unamended soil after 28 d. However, there were subtle reductions in the extents of ^{14}C-
211 naphthalene mineralisation in the aged soil (Table 3) compared to freshly spiked soil
212 (Table 2).
213
214 *3.2 Mineralisation of ^{14}C-phenanthrene in soils amended with rhizosphere soil or root*
215 *tissues*
216 Mineralisation of ^{14}C-phenanthrene was monitored in freshly spiked and 28 d aged
217 soils amended with rhizosphere soil and root tissues using respirometric assays (Figure
218 2; Tables 2–3). The indigenous soil microflora in contaminated soils amended with
219 rhizosphere soil or root tissues rapidly mineralised phenanthrene as indicated by the
220 relatively short lag phases (Tables 2–3). The lag phases prior to extensive
221 mineralisation of ^{14}C-phenanthrene (≥ 5% added ^{14}C–activity) ranged from 30.20±0.20
222 h (i.e. chanel grass root tissues) to 147.06±2.17 h (i.e. unamanded soil) at 0 d, and from
223 4.38±0.14 (i.e. blackberry root tissues) to 6.00±0.27 h (i.e. reed canry grass root
224 tissues) after 28 d. The lag phases in soils amended with root tissues and rhizosphere
225 soil were significantly ($P < 0.001$) shorter compared to unamended soil. After 28 d
226 soil–contaminant contact time, there were no ($P > 0.05$) significant differences in the
227 lag phases between amended treatments and unamended soil. Soils amended with
228 chanel grass root tissues exhibited the shortest ($P < 0.001$) lag phase in relative to other
229 treatments at 0 d. The rates of ^{14}C-phenanthrene mineralisation were significantly ($P <$
230 0.001) faster in soils amended with rhizosphere soil and root tissues compared to the
231 unamended soil at 0 d (Table 2). After 28 d ageing period, there were no significant (P

232 > 0.05) differences in the rates of ^{14}C-phenanthrene mineralisation in soil amended
233 with rhizosphere soil or root tissues and unamended soil.
234 The extents of ^{14}C-phenanthrene mineralisation ranged from 36.95±1.20 to
235 44.28±1.80% in soils amended with rhizosphere soil; from 40.47±0.21 to 43.54±1.91%
236 in soils amended with root tissues; and 28.78±0.63% in the unamended soil at 0 d
237 (Figure 2; Table 2). Enhanced extents of ^{14}C-phenanthrene mineralisation were
238 observed in soils amended with rhizosphere soil or root tissues ($P < 0.001$) relative to
239 unamended soil. In a direct comparison, there were no significant ($P > 0.05$)
240 differences in the extents of ^{14}C-phenanthrene mineralisation in soils amended with
241 rhizosphere soil or root tissues at 0 d. After 28 d soil–contaminat contact time,
242 mineralisation ranged from 43.23±1.16 to 46.19±1.49% in soils amended with
243 rhizosphere soil; from 42.98±1.01 to 47.24±1.30% in soils amended with root tissues;
244 and 45.15±0.87% in the unamended soil (Figure 2; Table 3). There were no significant
245 ($P > 0.05$) differences in levels of ^{14}C-phenanthrene mineralisation in amended soils
246 and unamended soil between 0 and 28 d time points.
247
248 *3.3 Mineralisation of ^{14}C-hexadecane in soils amended with rhizosphere soil and root*
249 *tissues*
250 Mineralisation of ^{14}C-hexadecane was monitored in freshly spiked and 28 d aged soils
251 amended with rhizosphere soil and root tissues using respirometric assays (Figure 3;
252 Tables 2–3). The indigenous soil microflora in contaminated soils amended with
253 rhizosphere soil or root tissues showed slow degradative capabilities as indicated by the
254 long lag phases (Tables 2–3). The lag phases prior to extensive mineralisation of ^{14}C-
255 hexadecane (≥ 5% added ^{14}C-activity) ranged from 28.59±3.44 (i.e. reed canary grass
256 root tissues) to 50.42±4.86 h (i.e. goat willow rhizosphere soil) at 0 d and from

257 5.08±0.21 (i.e. chanel grass root tissues) to 9.21±1.34 h (i.e. goat willow root tissues)
258 after 28 d. With exception of blackberry root tissues, reed canary grass rhizosphere soil
259 and tissues amendments, the lag phases in soils amended with rhizosphere soil or root
260 tissues were significantly ($P < 0.05$) shorter compared to unamended soil. After 28 d
261 soil–contaminant contact time, there were no ($P > 0.05$) significant differences in the
262 lag phases between amended soils and unamended soil. However, soil amended with
263 canary grass rhizopshere soil exhibited the shortest lag phase compared to the other
264 treatments at 28 d. The rates of ^{14}C-hexadecane mineralisation were not significantly
265 ($P > 0.05$) different in soils amended with reed canary grass rhizosphere soil or
266 blackberry root tissues compared to the unamended soil at 0 d (Table 2). With the
267 exception of soils amended with reed canary grass rhizosphere soil or blackberry root
268 tissues, there were reductions in rates of ^{14}C-hexadecane mineralisation in other
269 amended soils relative to the unamended soil. After 28 d ageing period, there were no
270 significant ($P > 0.05$) differences in the rates of ^{14}C-hexadecane mineralisation in soils
271 amended with rhizosphere soil or root tissues and the unamended soil.
272 The extents of ^{14}C-hexadecane mineralisation ranged from 33.87±1.88 to 42.93±1.64%
273 in soils amended with rhizosphere soil; from 36.73±1.75 to 39.70±1.15% in soils
274 amended with root tissues; and 35.27±2.07% in the unamended soil at 0 d (Figure 3;
275 Table 2). The extents of ^{14}C-hexadecane mineralisation in soils amended with
276 rhizosphere soil or root tissues were not significantly ($P > 0.05$) different relative to
277 unamended soil at 0 d. After 28 d soil–contaminat contact time, mineralisation ranged
278 from 33.32±1.80 to 39.88±1.43% in soils amended with rhizosphere soil; 37.41±0.43
279 to 40.54±1.01% in soils amended with root tissues; and 33.39±1.37% in unamended
280 soil (Figure 3; Table 3). There were no significant ($P > 0.05$) differences in ^{14}C-
281 hexadecane mineralisation between amended soils and unamended soil after 28 d.

282 Overall, these results showed that contaminated soils amended with rhizosphere soil or
283 root tissues did not enhance ^{14}C-hexadecane mineralisation by indigenous soil
284 microflora.
285
286 *3.4 Mineralisation of ^{14}C-octacosane in soils amended with rhizosphere soil or root*
287 *tissues*
288 Mineralisation of ^{14}C-octacosane was monitored in freshly spiked and 28 d aged soils
289 amended with rhizosphere soil or root tissues using respirometric assays (Figure 4;
290 Tables 2–3). The indigenous soil microflora in contaminated soils amended with
291 rhizosphere soil or root tissues showed slow degradative capacities as indicated by the
292 long lag phases (Tables 2–3). The lag phases prior to extensive mineralisation of ^{14}C-
293 octacosane ($\geq$ 5% added ^{14}C-activity) ranged from 28.02±2.07 (i.e. reed canary grass
294 root tissues) to 68.99±2.32 h (i.e. blackberry root tissues) at 0 d, and from 27.63±1.22
295 (i.e. blackberry rhizosphere soil) to 51.77±3.07 h (i.e. blackberry root tissues) after 28
296 d. With the exception of reed canary grass root tissues amendment, the lag phases in
297 soils amended with rhizosphere soil or root tissues were not significantly ($P > 0.05$)
298 reduced compared to the unamended soil at 0 d. However, soil amended with
299 blackberry root tissues exhibited the longest lag phase compared to the other treatments
300 at 0 d. With the exception of blackberry and goat willow root tissues amendments, the
301 lag phases in soils amended with rhizosphere soil or root tissues were not significantly
302 ($P > 0.05$) different compared to unamended soil after 28 d. The rates of ^{14}C-
303 octacosane mineralisation in soils amended with rhizosphere soil were not significantly
304 ($P > 0.05$) different compared to unamended soil at 0 d (Table 2). With the exception
305 of soil amended with goat willow root tissues, faster rates ($P < 0.05$) of ^{14}C-octacosane
306 mineralisation were measured in contaminated soils amended with root tissues

307 compared to the unamended soil at 0 d. After 28 d contact time, the rates of ^{14}C-
308 octacosane mineralisation were not significantly ($P > 0.05$) different in soils amended
309 with rhizosphere soil or root tissues compared to the unamended soil (Table 2).
310 Although there were subtle differences in the rates of ^{14}C-octacosane mineralisation in
311 soil following 28 d ageing period, there were no significant ($P > 0.05$) differences in
312 the mineralisation rates between the amended soils and the unamended soil.
313 The extents of ^{14}C-octacosane mineralisation ranged from 31.98±1.71 to 38.42±2.02%
314 in soils amended with rhizosphere soil; from 32.20±1.43 to 43.75±0.40% in soils
315 amended with root tissues; and 36.88±0.42% in the unamended soil at 0 d (Figure 4;
316 Table 2). The extents of ^{14}C-octacosane mineralisation in soils amended with
317 rhizosphere soil or root tissues were not significantly ($P > 0.05$) different relative to
318 unamended soil at 0 d. However, the highest extent of mineralisation (43.75±0.40%)
319 was observed in soil amended with reed canary grass root tissues, while the lowest
320 extent of mineralisation (31.98±1.71%) was measured in soil amended with chanel
321 grass rhizosphere soil at 0 d. After 28 d soil–contaminat contact time, mineralisation
322 ranged from 38.01±1.06 to 39.12±0.99% in soils amended with rhizosphere soil; from
323 36.28±0.96 to 43.92±0.65% in soils amended with root tissues; and 34.21±0.50% in the
324 unamended soil (Figure 4; Table 3). There were no significant ($P > 0.05$) differences in
325 the extents of ^{14}C-octacosane mineralisation between amended soils and unamended
326 soil after 28 d. Overall, soils amended with rhizosphere soil or root tissues consistently
327 displayed similar patterns of ^{14}C-octacosane and ^{14}C-hexadecane mineralisation.
328
329 *3.5 Changes in numbers of heterotrophic and hydrocarbon-degrading microbes*
330 The numbers of total heterotrophic bacteria (THB) and hydrocarbon-degrading
331 microbes in the contaminated soils amended with rhizosphere soil or root tissues were

enumerated by standard microbiological techniques (Tables 4–5). At 0 d time point, the numbers of indigenous microbes in the amended soil ranged from 10^6 to 10^8 CFUs g^{-1} for THB and from 10^6 to 10^9 CFUs g^{-1} for hydrocarbon-degrading microbes (Table 4). Microbial cell numbers for THB and hydrocarbon-degrading microbes of 10^6 CFUs g^{-1} were measured in the unamended soil. The CFUs g^{-1} for THB and hydrocarbon-degrading microbes in the amended soils were in similar ranges after 28 d contact time compared to 0 contact time. Although the CFUs g^{-1} for THB and hydrocarbon-degrading microflora in unamended soil remained similar, the CFUs g^{-1} for hydrocarbon degraders in soils amended with rhizosphere soil or root tissues significantly ($P < 0.001$) increased following 28 d ageing period (Table 5). The highest numbers of hydrocarbon-degrading microbes $>10^9$ CFUs g^{-1} were measured in root tissues-amended soils after 28 d, and the microbial cell numbers in the amended soil or unamended soil increased following pre-exposure of soil to contaminants.

4. Discussion

4.1 Development of ^{14}C-PAH and ^{14}C-aliphatic hydrocarbon mineralisation in soils amended with rhizosphere soil or roots tissues

Root exudates, including organic compounds which are analogues of PAHs, may serve as nutrient sources for microbial growth and can stimulate the biodegradation of organic contaminants in soil. For example, monoterpenes have been shown to stimulate the biodegradation of 2,4-dichlorophenol by indigenous soil microorganisms (Rhodes et al., 2007; McLoughlin et al., 2009). Several studies have linked increased hydrocarbon degradation in soil to plant root exudates and increases in rhizosphere-associated microbial communities (Knaebel and Vestal, 1992; Gunther et al., 1996; Kawasaki et al., 2011; Phillips et al., 2012). This current study investigated the impact

357 of rhizosphere soil or root tissues of reed canary grass, channel grass, blackberry and
358 goat willow on the biodegradation of target ^{14}C-hydrocarbons in amended soils. The
359 plants, rhizosphere soil or root tissues used in this study were sampled from the former
360 Shell ICI Refinery site. The addition of rhizosphere soil or root tissues significantly
361 enhanced ^{14}C-PAHs (phenanthrene and naphthalene), but did not stimulate ^{14}C-
362 aliphatic hydrocarbons (hexadecane or octacosane) mineralisation in freshly amended
363 soils. This may be attributed to the fact that root exudates may influence the
364 degradative capacity of an existing microbial community via a number of mechanisms,
365 including shifts in catabolic gene expression, general metabolic status, and/or catabolic
366 gene transfer (Van Elsas et al., 2003; Da Silva et al., 2006). Although the precise
367 mechanism for this enhanced biodegradation remains unclear, the catabolic activity and
368 degradative potential in the amended soils could have been enhanced through
369 physiochemical and biological changes caused by organic compounds in the root
370 exudates.
371 The enhanced mineralisation of PAHs by indigenous soil microbes in soils freshly
372 amended with rhizopshere soil or root tissues may be attributed to a combination of
373 mechanisms rather than one single mechanism. In this study, contaminated soil
374 amended with root tissues of reed canary grass, blackberry or goat willow exhibited
375 greater extents of mineralisation. The addition of rhizosphere soil or root tissues might
376 have provided nutrient substrates for microbial growth and stimulated the desired
377 microbial catabolic capabilities in the freshly contaminated soil. It is possible that this
378 was complicated by substrate interactions such as simultaneous biomass growth on
379 multiple substrates (Guha et al., 1999). PAH–analogous in the root exudates might
380 have stimulated appropriate enzymatic pathways for microbial mineralisation of the
381 PAHs in soil, and the results support the findings of Miya and Firestone (2001).

382 Changes in microbial cell numbers corresponded with enhanced microbial activity and
383 growth of indigenous hydrocarbon-degrading microbes. This corresponds to the higher
384 number of the hydrocarbon degraders enumerated in the amended soils and can be
385 attributed to the microbes which might have been introduced with the rhizosphere soil
386 or root tissues. It has been observed that root exudates supply organic compounds that
387 serve as co–metabolites in microbial organic contaminant degradation or cometabolic
388 biotransformation (Haby and Crowley, 1996; Hegde and Fletcher, 1996). The addition
389 of ground hybrid poplar roots produced a 165% increase in atrazine mineralisation
390 (Burken and Schnoor, 1996) and this was primarily attributed to dehalogenase enzymes
391 in the root tissues that have the capability to degrade atrazine (Schnoor et al., 1995).
392 Enhanced degradation through cometabolism of benzo[a]pyrene by the rhizobacterium
393 *Sphingomonas yanoikuyae* JAR02 *in vitro* in the presence of root extracts obtained
394 from plant species, including mulberry (*Morus alba*) and hybrid willow (*Salix alba x*
395 *matsudana*) has been reported by Rentz et al., (2005). A further explanation could be
396 attributed to the effect of some of the bioactive compounds, such as alkaloids,
397 flavonoids, tannins, saponins, phenols and/or cocktails of several other phytochemicals
398 exuded from plant roots (Salt et al., 1998). In addition to root exudates that support the
399 growth and activities of rhizosphere-associated microbes, plant exudates may
400 contribute to the enhanced mineralisation of organic contaminants through an increase
401 in microbial density (more than 1–3 orders of magnitude than in non-vegetated or bulk
402 soil), diversity and/or metabolic activity (Azaizeh et al., 2010).
403 Although the degree of enzymes release into soils and sediments remains poorly
404 understood (Schnoor et al., 1995), the presence of phytochemical compounds in
405 rhizosphere soil or root tissues in sufficient quantities might have primed specific
406 biodegradation activities or promote selective degradation capacity of the indigenous

407 soil microbes. However, it is widely accepted that the rates and extents of the
408 biodegradation of PAHs differ as a result of physico-chemical properties, such as
409 molecular size, structure, hydrophobicity and solubility (Stokes et al., 2005). In this
410 study, ^{14}C-aliphtic hydrocarbon mineralisation in soil amended with rhizosphere soil or
411 root tissues may be due to the preferential or co-utilisation of the additional carbon
412 supplied in the amendments over the target substrate. It has been reported that aliphatic
413 and aromatic hydrocarbons, such as flavonoids (Siciliano and Germida, 1998), and
414 phenanthrene derivatives, such as retene and nudol (Bhandari et al., 1985), occur
415 naturally in plant materials. It is possible that plant organic compounds from the
416 amendments influenced soil microbial activity by providing co-substrates for biomass
417 growth (Guha et al., 1999). According to Read et al. (2003), plant roots release
418 phospholipid surfactants that modify the physical and chemical properties of soil. In
419 this study, associated organic compounds from rhizosphere soil or root tissues
420 amendments might have affected the solubilty and/or bioavailability of aliphatic
421 hydrocarbons during the slurry biodegradation. However, alkanes such as *n*-
422 hexadecane have a log K_{ow} of approximately 9.1, a reported solubility of up to 0.0263
423 mg l^{-1} (Bai et al., 1998), and as discrete compounds are unlikely to be effectively
424 mineralised by the indigenous soil microbes. Pre-exposure of soil microorganisms to
425 organic contaminants, but not amendment with rhizosphere soil or root tissues,
426 appeared to be the main factor that affected ^{14}C-aliphatic hydrocarbon mineralisation.
427
428 *4.2 Conclusions*
429 This study showed that the addition of rhizosphere soil or root tissues of four different
430 plant species significantly enhanced ^{14}C-PAHs mineralisation, but did not stimulate
431 mineralisation of ^{14}C-aliphatic hydrocarbons in freshly contaminated soils. This current

study provide further understanding of enhanced mineralisation of PAHs in rhizosphere soil, and the biodegradation of PAHs could be enhanced through organic coompounds from rhizosphere soil or root tissues. This study further confirmed the finding of Stroud et al. (2007), that PAHs with lower molecular weights (LMW) may be mineralised faster than those with higher molecular weights (HMW) as LMW-PAHs are more suitable as sole carbon source to the microbial communities. Although pre-exposure of soil microorganisms to hydrocarbons decreased the lag phases and increased the initial rates of mineralisation, addition of plant root-exuded chemicals may have practical application for remediation of petroleum-contaminated soils. Further research is required to identify the specific root exudate components which enhance hydrocarbons mineralisation in soil.

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589
590

591 **Tables**

592 Table 1: Physicochemical and microbial characteristics of Myerscough soil; values are

593 the mean (n = 3) ± standard errors of the mean (SEM).

Parameter		Value
pH in (dH_2O)		6.50 ± 0.08
Moisture content (%)		34.87 ± 0.89
Maximum water holding capacity (%)		38.03 ± 0.02
Elemental analysis		
	Total extractable organic carbon (%)	1.65 ± 0.01
	Total extractable carbon (%)	1.70 ± 0.09
	Total extractable nitrogen (%)	0.14 ± 0.01
	Soil organic matter (%)	2.71 ± 0.04
	Phosphorus (μg g^{-1})	997.00 ± 0.01
	C:N ratios	11.8:1
Particle analysis		
	Clay (%)	19.5 ± 0.70
	Silt (%)	20.0 ± 0.90
	Sand - Total (%)	60.4 ± 1.40
	Coarse sand	0.12 ± 0.01
	Medium sand	6.90 ± 0.10
	Fine sand	53.30 ± 0.60
Microbial analysis		
	Heterotrophs (CFU g^{-1})	$5.28 \times 10^4 \pm 0.00 \times 10^0$
	Hexadecane degraders (CFU g^{-1})	$6.24 \times 10^4 \pm 3.33 \times 10^4$
	Octacosane degraders (CFU g^{-1})	$3.05 \times 10^4 \pm 0.00 \times 10^0$
	Phenanthrene degraders (CFU g^{-1})	$4.04 \times 10^4 \pm 3.33 \times 10^4$
	Naphthalene degraders (CFU g^{-1})	$5.14 \times 10^4 \pm 3.33 \times 10^4$

594

595 Table 2: Mineralisation of ^{14}C–hydrocarbons by indigenous soil microflora in 0 d contaminated soil amended with 5% wet weight (A)

596 rhizosphere or (B) root tissue during 14 d respirometric assays. Values are the mean (n = 3) ± standard error of the mean (SEM).

	Treatment conditions	Lag phase (h)			Maximum rate (% h^{-1})			Cumulative extents (%)		
		Unamended Soil (Control)	Rhizosphere treatment	Root tissue treatment	Unamended Soil (Control)	Rhizosphere treatment	Root tissue treatment	Unamended Soil (Control)	Rhizosphere treatment	Root tissue treatment
Naphthalene	Bulk soil	72.20 ± 0.04			0.87 ± 0.05			36.31 ± 1.51		
	Reed canary grass		26.93 ± 0.04	3.27 ± 0.18		1.35 ± 0.03	1.54 ± 0.08		46.12 ± 1.76	59.24 ± 1.55
	Chanel grass		26.81 ± 0.83	4.79 ± 1.02		1.19 ± 0.07	1.21 ± 0.19		42.79 ± 2.32	51.82 ± 1.72
	Blackberry		26.69 ± 0.08	4.29 ± 0.08		1.54 ± 0.05	1.17 ± 0.02		56.28 ± 1.75	57.32 ± 2.06
	Goat willow		26.92 ± 0.03	25.54 ± 0.12		1.48 ± 0.02	1.70 ± 0.03		55.46 ± 1.89	58.31 ± 0.16
Phenanthrene	Bulk soil	147.06 ± 2.17			0.24 ± 0.02			28.78 ± 0.63		
	Reed canary grass		76.74 ± 0.29	80.83 ± 2.52		0.81 ± 0.03	0.70 ± 0.15		37.43 ± 1.75	42.98 ± 0.45
	Chanel grass		54.27 ± 0.12	30.72 ± 0.20		0.68 ± 0.01	0.68 ± 0.02		41.23 ± 1.75	40.47 ± 0.21
	Blackberry		86.08 ± 2.39	76.42 ± 0.32		0.75 ± 0.03	0.85 ± 0.06		36.95 ± 1.20	43.47 ± 2.08
	Goat willow		101.58 ± 0.04	94.13 ± 2.35		0.71 ± 0.01	1.04 ± 0.09		44.28 ± 1.80	43.54 ± 1.91
Hexadecane	Bulk soil	31.69 ± 0.69			0.47 ± 0.04			35.27 ± 2.07		
	Reed canary grass		30.79 ± 0.69	28.59 ± 3.44		0.49 ± 0.05	0.22 ± 0.02		42.93 ± 1.64	37.27 ± 2.07
	Chanel grass		47.19 ± 0.24	49.54 ± 1.16		0.32 ± 0.07	0.39 ± 0.07		38.61 ± 1.92	36.73 ± 1.75
	Blackberry		40.22 ± 3.69	29.53 ± 1.70		0.26 ± 0.04	0.45 ± 0.02		36.57 ± 0.48	39.70 ± 1.15
	Goat willow		50.42 ± 4.86	37.94 ± 1.65		0.30 ± 0.01	0.26 ± 0.03		33.87 ± 1.88	37.69 ± 1.89
Octacosane	Bulk soil	34.55 ± 2.51			0.25 ± 0.02			36.88 ± 0.42		
	Reed canary grass		52.21 ± 5.62	28.02 ± 2.07		0.27 ± 0.01	0.44 ± 0.01		38.42 ± 2.02	43.75 ± 0.40
	Chanel grass		41.81 ± 3.82	49.86 ± 1.15		0.27 ± 0.02	0.35 ± 0.01		30.20 ± 1.93	38.05 ± 1.47
	Blackberry		47.63 ± 2.68	68.99 ± 2.32		0.26 ± 0.01	0.43 ± 0.01		31.98 ± 1.71	37.40 ± 1.29
	Goat willow		47.69 ± 2.52	36.14 ± 0.69		0.28 ± 0.01	0.32 ± 0.03		33.84 ± 1.95	32.20 ± 1.43

597 Table 3: Mineralisation of ^{14}C–hydrocarbons by indigenous soil microflora in 28 d contaminated soil amended with 5% (wet weight) (A)

598 rhizosphere or (B) root tissue during 14 d respirometric assays. Values are the mean (n = 3) ± standard error of the mean (SEM).

	Treatment conditions	Lag phase (h)			Maximum rate (% h^{-1})			Cumulative extents (%)		
		Unamended Soil (Control)	Rhizosphere treatment	Root tissue treatment	Unamended Soil (Control)	Rhizosphere treatment	Root tissue treatment	Unamended Soil (Control)	Rhizosphere treatment	Root tissue treatment
Naphthalene	Bulk soil	3.86 ± 0.00			1.36 ± 0.07			46.39 ± 1.77		
	Reed canary grass		3.42 ± 0.10	4.42 ± 0.18		1.47 ± 0.04	1.13 ± 0.05		47.96 ± 1.49	49.39 ± 1.77
	Chanel grass		4.15 ± 0.06	3.58 ± 0.04		1.24 ± 0.04	1.37 ± 0.03		44.20 ± 1.38	51.86 ± 1.19
	Blackberry		3.66 ± 0.05	3.83 ± 0.07		1.37 ± 0.02	1.31 ± 0.02		52.47 ± 1.72	48.96 ± 1.18
	Goat willow		3.61 ± 0.01	4.05 ± 0.02		1.38 ± 0.00	1.23 ± 0.00		51.78 ± 1.12	52.31 ± 0.84
Phenanthrene	Bulk soil	4.52 ± 0.10			1.10 ± 0.02			45.15 ± 0.87		
	Reed canary grass		5.45 ± 0.28	6.00 ± 0.27		0.92 ± 0.05	0.84 ± 0.04		43.23 ± 1.16	42.98 ± 1.01
	Chanel grass		4.98 ± 0.02	4.72 ± 0.25		1.01 ± 0.00	1.08 ± 0.04		44.16 ± 0.74	49.68 ± 1.81
	Blackberry		4.50 ± 0.11	4.38 ± 0.14		1.11 ± 0.03	1.14 ± 0.04		46.58 ± 1.83	47.24 ± 1.30
	Goat willow		4.48 ± 0.03	4.82 ± 0.04		1.11 ± 0.01	1.07 ± 0.03		46.19 ± 1.49	46.21 ± 0.95
Hexadecane	Bulk soil	5.55 ± 0.07			0.90 ± 0.01			33.65 ± 1.37		
	Reed canary grass		4.66 ± 0.12	5.20 ± 0.07		1.07 ± 0.03	0.96 ± 0.01		38.68 ± 1.10	40.54 ± 1.01
	Chanel grass		4.83 ± 0.03	5.08 ± 0.21		1.03 ± 0.01	0.96 ± 0.01		39.88± 1.43	39.47 ± 1.05
	Blackberry		5.61 ± 0.31	7.35 ± 0.21		0.90 ± 0.05	0.68 ± 0.03		35.49 ± 1.68	37.41 ± 0.43
	Goat willow		5.63 ± 0.14	9.21 ± 1.34		0.85 ± 0.04	0.59 ± 0.06		33.32 ± 1.80	38.62 ± 1.11
Octacosane	Bulk soil	36.36 ± 0.14			0.20 ± 0.01			34.21 ± 0.50		
	Reed canary grass		31.54 ± 0.55	32.85 ± 1.99		0.23 ± 0.02	0.30 ± 0.02		38.01 ± 1.06	43.92 ± 0.65
	Chanel grass		30.14 ± 0.48	28.43 ± 0.62		0.24 ± 0.02	0.30 ± 0.01		39.12 ± 0.99	43.37 ± 0.67
	Blackberry		27.63 ± 1.22	51.77 ± 3.07		0.23 ± 0.01	0.29 ± 0.05		38.17 ± 1.01	42.76 ± 2.38
	Goat willow		28.83 ± 1.20	44.24 ± 1.19		0.24 ± 0.04	0.25 ± 0.03		38.62 ± 1.29	36.28 ± 0.96

599

600 Table 4: Microbial cell numbers of total heterotrophic and hydrocarbon degrading microbes during ^{14}C–hydrocarbons mineralisation in

601 soil amended with 5% (wet weight) rhizosphere or root tissue at 0 d. Values are the mean (n = 3) ± standard error of the mean (SEM).

	Treatment conditions	Microbial cell numbers (CFU g^{-1}) at 0 d				
		Degraders (Unamended soil)	Heterotrophs (Rhizosphere)	Degraders (Rhizosphere)	Heterotrophs (Root)	Degraders (Root)
Naphthalene	Bulk soil	$6.06 \times 10^6 \pm 0.00 \times 10^0$				
	Reed canary grass		$8.99 \times 10^6 \pm 3.33 \times 10^4$	$6.06 \times 10^6 \pm 0.00 \times 10^0$	$1.21 \times 10^8 \pm 0.00 \times 10^0$	$1.76 \times 10^9 \pm 1.53 \times 10^7$
	Chanel grass		$8.99 \times 10^6 \pm 3.33 \times 10^4$	$8.99 \times 10^7 \pm 3.33 \times 10^5$	$3.00 \times 10^8 \pm 5.77 \times 10^5$	$9.09 \times 10^8 \pm 0.00 \times 10^0$
	Blackberry		$1.49 \times 10^7 \pm 3.33 \times 10^4$	$6.06 \times 10^7 \pm 0.00 \times 10^0$	$6.06 \times 10^7 \pm 0.00 \times 10^0$	$9.09 \times 10^8 \pm 0.00 \times 10^0$
	Goat willow		$6.06 \times 10^7 \pm 0.00 \times 10^0$	$3.03 \times 10^7 \pm 0.00 \times 10^0$	$8.99 \times 10^8 \pm 3.33 \times 10^5$	$3.05 \times 10^9 \pm 3.33 \times 10^6$
Phenanthrene	Bulk soil	$5.56 \times 10^6 \pm 1.67 \times 10^4$				
	Reed canary grass		$8.99 \times 10^6 \pm 3.33 \times 10^4$	$5.96 \times 10^6 \pm 3.33 \times 10^4$	$1.21 \times 10^8 \pm 0.00 \times 10^0$	$9.09 \times 10^8 \pm 0.00 \times 10^0$
	Chanel grass		$8.99 \times 10^6 \pm 3.33 \times 10^4$	$8.99 \times 10^7 \pm 3.33 \times 10^5$	$3.00 \times 10^8 \pm 5.77 \times 10^5$	$3.66 \times 10^9 \pm 3.33 \times 10^6$
	Blackberry		$1.49 \times 10^7 \pm 3.33 \times 10^4$	$5.96 \times 10^7 \pm 3.33 \times 10^5$	$6.06 \times 10^7 \pm 0.00 \times 10^0$	$3.15 \times 10^9 \pm 3.00 \times 10^7$
	Goat willow		$6.06 \times 10^7 \pm 0.00 \times 10^0$	$9.09 \times 10^7 \pm 5.77 \times 10^7$	$8.99 \times 10^7 \pm 3.33 \times 10^5$	$7.07 \times 10^8 \pm 6.67 \times 10^6$
Hexadecane	Bulk soil	$3.33 \times 10^6 \pm 5.77 \times 10^4$				
	Reed canary grass		$8.99 \times 10^6 \pm 3.33 \times 10^4$	$6.06 \times 10^7 \pm 0.00 \times 10^0$	$1.21 \times 10^8 \pm 0.00 \times 10^0$	$1.52 \times 10^9 \pm 5.77 \times 10^6$
	Chanel grass		$8.99 \times 10^6 \pm 3.33 \times 10^4$	$8.99 \times 10^7 \pm 3.33 \times 10^5$	$3.00 \times 10^8 \pm 5.77 \times 10^5$	$9.09 \times 10^8 \pm 0.00 \times 10^0$
	Blackberry		$1.49 \times 10^7 \pm 3.33 \times 10^4$	$9.09 \times 10^7 \pm 0.00 \times 10^0$	$6.06 \times 10^7 \pm 0.00 \times 10^0$	$9.09 \times 10^8 \pm 5.77 \times 10^6$
	Goat willow		$6.06 \times 10^7 \pm 0.00 \times 10^0$	$5.86 \times 10^6 \pm 8.82 \times 10^5$	$8.99 \times 10^7 \pm 3.33 \times 10^5$	$6.26 \times 10^8 \pm 0.00 \times 10^0$
Octacosane	Bulk soil	$6.89 \times 10^6 \pm 8.82 \times 10^4$				
	Reed canary grass		$8.99 \times 10^6 \pm 3.33 \times 10^4$	$6.06 \times 10^7 \pm 0.00 \times 10^0$	$1.21 \times 10^8 \pm 0.00 \times 10^0$	$3.04 \times 10^9 \pm 3.33 \times 10^6$
	Chanel grass		$8.99 \times 10^6 \pm 3.33 \times 10^4$	$9.09 \times 10^9 \pm 0.00 \times 10^0$	$3.00 \times 10^8 \pm 5.77 \times 10^5$	$9.39 \times 10^8 \pm 5.77 \times 10^6$
	Blackberry		$1.49 \times 10^7 \pm 3.33 \times 10^4$	$1.05 \times 10^8 \pm 6.67 \times 10^5$	$6.06 \times 10^7 \pm 0.00 \times 10^0$	$9.09 \times 10^8 \pm 0.00 \times 10^0$
	Goat willow		$6.06 \times 10^7 \pm 0.00 \times 10^0$	$3.03 \times 10^7 \pm 0.00 \times 10^0$	$8.99 \times 10^7 \pm 3.33 \times 10^5$	$9.80 \times 10^8 \pm 8.82 \times 10^6$

602 Table 5: Microbial cell numbers of total heterotrophic and hydrocarbon degrading microbes during ^{14}C–hydrocarbons mineralisation in

603 soil amended with 5% (wet weight) rhizosphere or root tissue at 28 d. Values are the mean (n = 3) ± standard error of the mean (SEM).

	Treatment conditions	Microbial cell numbers (CFU g^{-1}) at 28 d				
		Degraders (Unamended soil)	Heterotrophs (Rhizosphere)	Degraders (Rhizosphere)	Heterotrophs (Root)	Degraders (Root tissue)
Naphthalene	Bulk soil	$7.58 \times 10^6 \pm 5.77 \times 10^4$				
	Reed canary grass		$1.04 \times 10^7 \pm 6.67 \times 10^4$	$1.65 \times 10^8 \pm 3.33$ E5	$1.28 \times 10^8 \pm 3.33 \times 10^5$	$1.89 \times 10^9 \pm 1.20 \times 10^7$
	Chanel grass		$1.03 \times 10^7 \pm 5.77 \times 10^4$	$1.03 \times 10^8 \pm 5.77$ E5	$2.98 \times 10^8 \pm 1.67 \times 10^6$	$9.80 \times 10^8 \pm 8.82 \times 10^6$
	Blackberry		$1.64 \times 10^7 \pm 5.77 \times 10^4$	$6.77 \times 10^7 \pm 3.33$ E5	$6.57 \times 10^7 \pm 1.67 \times 10^6$	$1.04 \times 10^9 \pm 3.33 \times 10^6$
	Goat willow		$7.07 \times 10^6 \pm 1.20 \times 10^5$	$5.25 \times 10^7 \pm 6.67$ E5	$8.99 \times 10^7 \pm 3.33 \times 10^5$	$3.00 \times 10^9 \pm 1.00 \times 10^7$
Phenanthrene	Bulk soil	$7.37 \times 10^6 \pm 3.33 \times 10^4$				
	Reed canary grass		$1.04 \times 10^7 \pm 6.67 \times 10^4$	$3.84 \times 10^7 \pm 8.82$ E5	$1.28 \times 10^8 \pm 3.33 \times 10^5$	$9.98 \times 10^8 \pm 3.33 \times 10^6$
	Chanel grass		$1.03 \times 10^7 \pm 5.77 \times 10^4$	$9.60 \times 10^7 \pm 8.82$ E5	$2.98 \times 10^8 \pm 1.67 \times 10^6$	$3.73 \times 10^9 \pm 1.53 \times 10^7$
	Blackberry		$1.64 \times 10^7 \pm 5.77 \times 10^4$	$6.46 \times 10^7 \pm 8.82$ E5	$6.57 \times 10^7 \pm 1.67 \times 10^6$	$3.27 \times 10^9 \pm 5.77 \times 10^6$
	Goat willow		$7.07 \times 10^6 \pm 1.20 \times 10^5$	$1.00 \times 10^8 \pm 5.77$ E5	$8.99 \times 10^7 \pm 3.33 \times 10^5$	$7.47 \times 10^8 \pm 3.33 \times 10^6$
Hexadecane	Bulk soil	$3.54 \times 10^6 \pm 3.33 \times 10^4$				
	Reed canary grass		$1.04 \times 10^7 \pm 6.67 \times 10^4$	$5.66 \times 10^7 \pm 3.33 \times 10^5$	$1.28 \times 10^8 \pm 3.33 \times 10^5$	$1.59 \times 10^9 \pm 8.82 \times 10^6$
	Chanel grass		$1.03 \times 10^7 \pm 5.77 \times 10^4$	$8.99 \times 10^7 \pm 3.33 \times 10^5$	$2.98 \times 10^8 \pm 1.67 \times 10^6$	$1.00 \times 10^9 \pm 5.77 \times 10^6$
	Blackberry		$1.64 \times 10^7 \pm 5.77 \times 10^4$	$9.90 \times 10^7 \pm 1.45 \times 10^6$	$6.57 \times 10^7 \pm 1.67 \times 10^6$	$9.44 \times 10^8 \pm 8.82 \times 10^6$
	Goat willow		$7.07 \times 10^6 \pm 1.20 \times 10^5$	$5.86 \times 10^7 \pm 8.82 \times 10^5$	$8.99 \times 10^7 \pm 3.33 \times 10^5$	$6.97 \times 10^8 \pm 5.77 \times 10^6$
Octacosane	Bulk soil	$7.07 \times 10^6 \pm 6.67 \times 10^4$				
	Reed canary grass		$1.04 \times 10^7 \pm 6.67 \times 10^4$	$6.06 \times 10^7 \pm 0.00$ E0	$1.28 \times 10^8 \pm 3.33 \times 10^5$	$3.12 \times 10^9 \pm 1.15 \times 10^7$
	Chanel grass		$1.03 \times 10^7 \pm 5.77 \times 10^4$	$9.80 \times 10^7 \pm 3.33$ E5	$2.98 \times 10^8 \pm 1.67 \times 10^6$	$9.80 \times 10^8 \pm 3.33 \times 10^6$
	Blackberry		$1.64 \times 10^7 \pm 5.77 \times 10^4$	$1.06 \times 10^8 \pm 5.77$ E5	$6.57 \times 10^7 \pm 1.6\,7 \times 10^6$	$9.70 \times 10^8 \pm 1.67 \times 10^6$
	Goat willow		$7.07 \times 10^6 \pm 1.20 \times 10^5$	$4.24 \times 10^7 \pm 5.77$ E5	$8.99 \times 10^7 \pm 3.33 \times 10^5$	$1.02 \times 10^9 \pm 8.82 \times 10^6$

604 **Figures**

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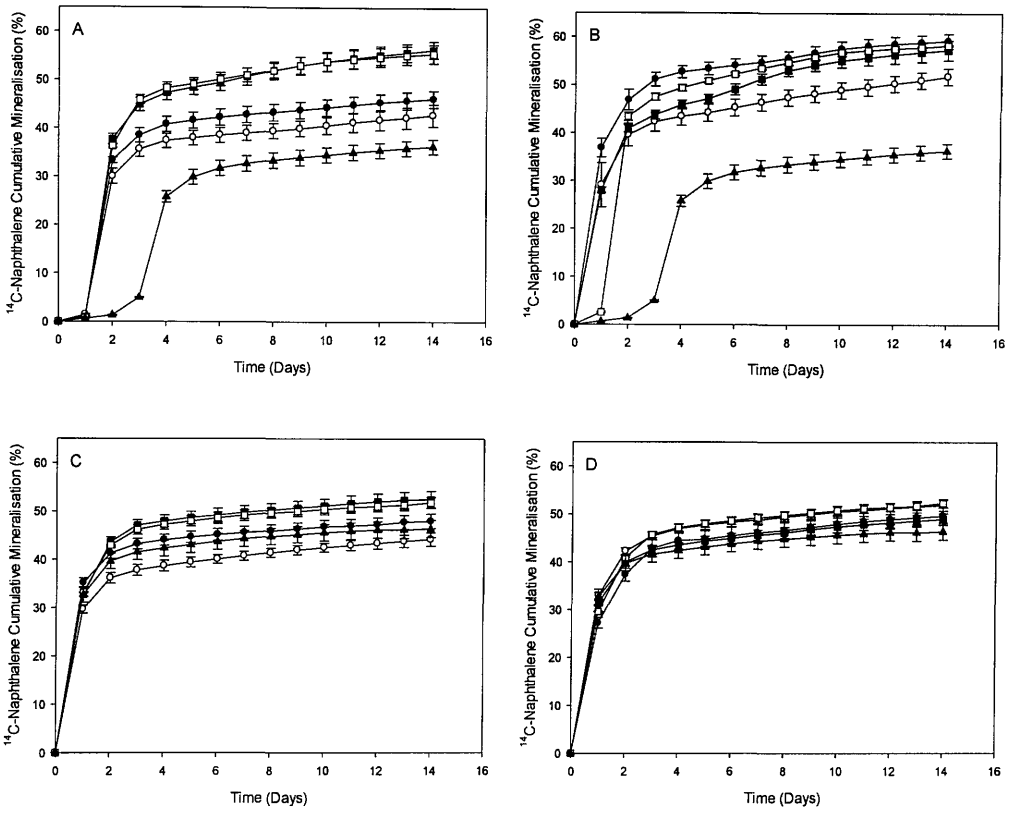

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608 Figure 1: Catabolism of 10 mg kg^{-1} naphthalene by indigenous soil microflora in
609 contaminated soil amended with 5% wet weight (A) rhizosphere or (B) root tissue at 0
610 d; and (C) rhizosphere or (D) root tissue after 28 d. Plant types: Reed Canary Grass
611 (●); Chanel Grass (○); Blackberry (■); Goat Willow (□) or unamended soil (control)
612 (▲). Data are presented as means ($n = 3$) and the error bars are the standard errors of
613 mean (SEM).

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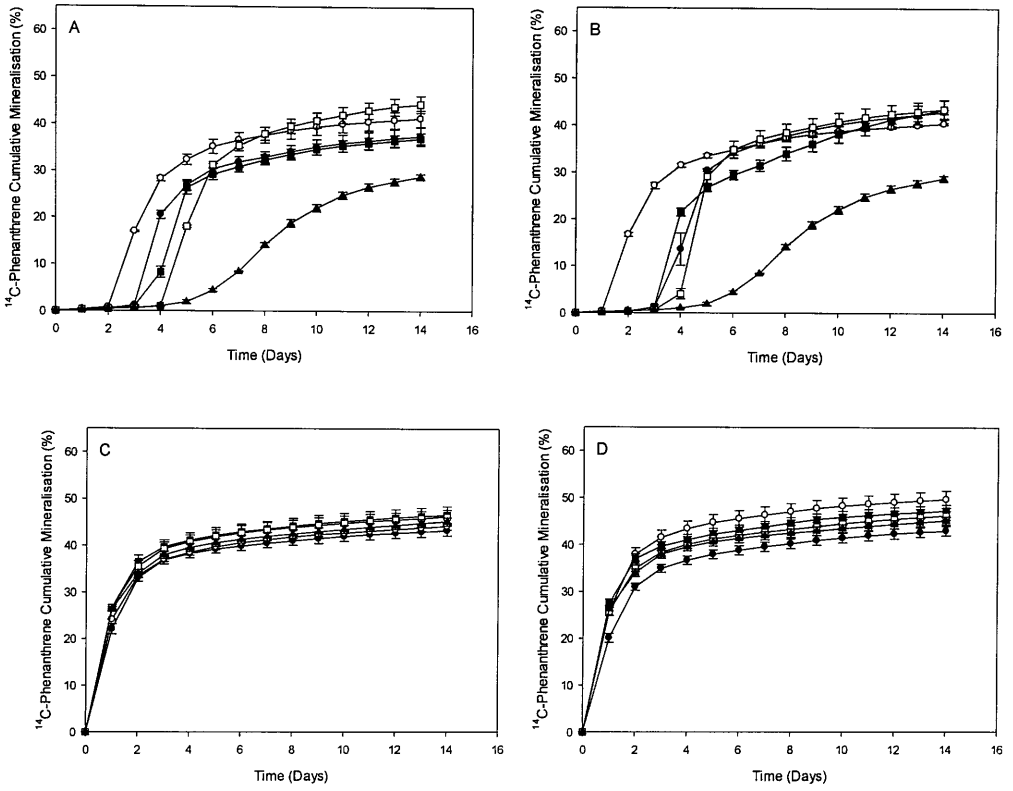

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618 Figure 2: Catabolism of 10 mg kg^{-1} phenanthrene by indigenous soil microflora in
619 contaminated soil amended with 5% wet weight (A) rhizosphere or (B) root tissue at 0
620 d; and (C) rhizosphere or (D) root tissue after 28 d. Plant types: Reed Canary Grass
621 (●); Chanel Grass (○); Blackberry (■); Goat Willow (□) or unamended soil (control)
622 (▲). Data are presented as means ($n = 3$) and the error bars are the standard errors of
623 mean (SEM).

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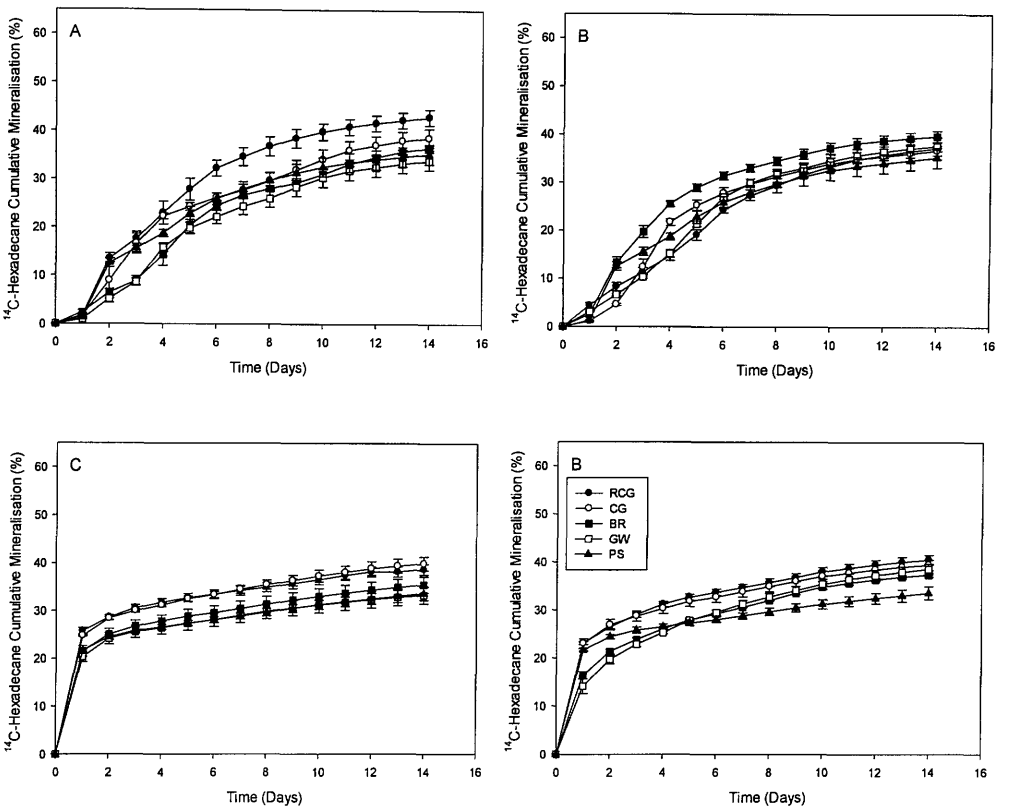

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628 Figure 3: Catabolism of 10 mg kg^{-1} hexadecane by indigenous soil microflora in
629 contaminated soil amended with 5% wet weight (A) rhizosphere or (B) root tissue at 0
630 d; and (C) rhizosphere or (D) root tissue after 28 d. Plant types: Reed Canary Grass
631 (●); Chanel Grass (○); Blackberry (■); Goat Willow (□) or unamended soil (control)
632 (▲). Data are presented as means ($n = 3$) and the error bars are the standard errors of
633 mean (SEM).

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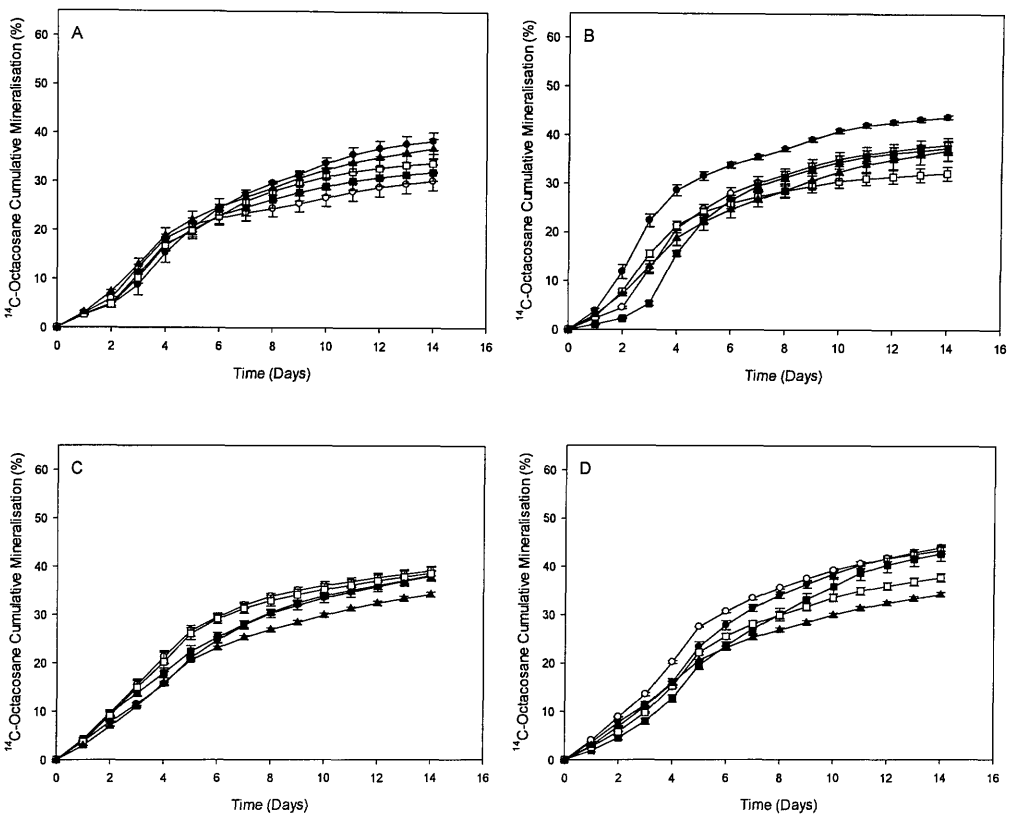

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638 Figure 4: Catabolism of 10 mg kg^{-1} octacosane by indigenous soil microflora in
639 contaminated soil amended with 5% wet weight (A) rhizosphere or (B) root tissue at 0
640 d; and (C) rhizosphere or (D) root tissue after 28 d. Treatment conditions: Reed Canary
641 Grass (●); Chanel Grass (○); Blackberry (■); Goat Willow (□) or unamended soil
642 (control) (▲). Data are presented as means ($n = 3$) and the error bars are the standard
643 errors of mean (SEM).

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