

Mineralisation of <sup>14</sup>C-phenanthrene in PAH-diesel contaminated soil: Impact of *Sorghum bicolor* and *Medicago sativa* mono- or mixed culture

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1 **Abstract**

2 Plant-assisted biodegradation can offer a cost-effective and sustainable approach for the  
3 bioremediation of PAHs in soil. As such, selecting the most appropriate plant species is  
4 important. The potential for plant-assisted biodegradation of complex PAH-diesel mixtures in  
5 soil by sorghum (*Sorghum bicolor*) and alfalfa (*Medicago sativa*) grown as monocultures and  
6 mixed cultures using <sup>14</sup>C-contaminants has not been widely reported. The objective of this  
7 study was to assess <sup>14</sup>C-phenanthrene mineralisation profiles in mixtures of PAH-diesel in soil  
8 in the presence of *Sorghum bicolor* and *Medicago sativa*. Soil was spiked with PAHs and  
9 diesel, after which *M. sativa* and *S. bicolor* were introduced and grown as mono- or mixed-  
10 cultures. The toxicity of the PAH-diesel oil mixture in the planted treatments, as well as its  
11 effect on the mineralisation of <sup>14</sup>C-phenanthrene were evaluated. Monocultures of both plant  
12 species tolerated the complex PAH-diesel mixtures based on growth and survival, and  
13 increased rates and extents of <sup>14</sup>C-phenanthrene mineralisation in soil. The influence of PAH  
14 concentration on <sup>14</sup>C-phenanthrene mineralisation profiles varied in planted and unplanted  
15 treatments. The rates and extents of <sup>14</sup>C-phenanthrene mineralisation tended to decrease in  
16 diesel amended soil, especially at low PAH concentrations. To the best of the authors'  
17 knowledge, this is the first report of <sup>14</sup>C-phenanthrene mineralisation patterns in complex PAH-  
18 diesel oil mixtures contaminated soil especially with respect to the specified plant species. The  
19 findings offer new insights on mono- and multi-species phytotoxicity as well as plant-assisted  
20 biodegradation of PAH mixtures in soil which may be useful in the risk assessment and  
21 remediation of contaminated sites.

22 Keywords: PAH mixtures; diesel oil amendment; Phytotoxicity; *Sorghum bicolor*; *Medicago*  
23 *sativa*; <sup>14</sup>C-phenanthrene mineralisation.

24

## 25 1. Introduction

26 Polycyclic aromatic hydrocarbons (PAHs) are hydrophobic organic contaminants with two  
27 or more fused benzene rings together. Generally, these compounds are of concern to human  
28 and environmental health due to their carcinogenicity, toxicity, and persistence in the  
29 environment (Juhasz and Naidu, 2000). The USEPA has classified 16 PAHs as priority  
30 pollutants including phenanthrene (Phe), benzo[a]anthracene (BaA), and benzo[a]pyrene  
31 (BaP) (USEPA, 2008). Although PAHs are released into the environment from natural  
32 combustion of organic matter, anthropogenic activities constitute the most important sources  
33 (Wilson and Jones, 1993). For example, burning of fossil fuel, coal, and wood, vehicular  
34 emissions, heating, and accidental spills of crude oil and other petroleum products among  
35 others are well known sources of PAH release into the environment.

36 Soil is considered a major sink for PAHs in the environment (Wild and Jones, 1995; Semple  
37 *et al.*, 2001). PAHs can be found as complex mixtures in soil, where they associate with other  
38 chemicals such as phenols, aliphatic hydrocarbons and metals (Allan *et al.*, 2007; Thavamani  
39 *et al.*, 2012). PAHs also exist in co-contamination with non-aqueous phase liquids (NAPLs)  
40 such as transformer oil from electrical cables and diesel oil from deliberate and accidental oil  
41 spillage around petroleum hydrocarbon contaminated sites (Molina-Barahona *et al.*, 2004).  
42 The implication is that co-contamination is likely to change the fate and behaviour of PAHs in  
43 soil (Lee *et al.*, 2003; Couling *et al.*, 2010). This effect has been previously observed under a  
44 range of conditions by different authors such as Swindell and Reid (2006) or Towell *et al.*  
45 (2011).

46 Considering the environmental implications of the presence of these contaminants in soil,  
47 various studies have reported the potential of plant-assisted biodegradation of PAHs in soil  
48 (Banks *et al.*, 2003; Meng *et al.*, 2011; Chen *et al.*, 2016; Deng and Zeng, 2017). Although the  
49 mechanisms promoting plant assisted biodegradation of PAHs and other hydrophobic organic  
50 contaminants are not fully understood, different processes have been observed to affect the  
51 biodegradation process (Oyelami *et al.*, 2013). Among these, plant identity (Panchenko *et al.*,  
52 2016) and root exudates have been hypothesised to play an important role (Fan *et al.*, 2008;

53 Wenzel, 2009; Gao *et al.*, 2017). Mixed cultures of two or more plant species can enhance  
54 rates and extents of biodegradation (Chen *et al.*, 2016), potentially due to nutrient- and  
55 metabolites-richer rhizosphere, when compared to their corresponding monocultures (Wenzel,  
56 2009). Since the effectiveness of plant-assisted biodegradation may differ with plant species  
57 (D'Orazio *et al.*, 2013), finding appropriate plant species mix may represent a confounding  
58 factor for phytoremediation (Panchenko *et al.*, 2016; Thijs *et al.*, 2017).

59 Plant-assisted biodegradation of complex PAH-diesel oil mixtures in soil, measured through  
60 a <sup>14</sup>C-PAH mineralisation approach, in the presence of mono- or mixed- cultures of *Medicago*  
61 *sativa* L. (Fabaceae) and *Sorghum bicolor* (L.) Moench (Poaceae) has not been previously  
62 reported. Plant-assisted biodegradation in this present study was used to imply increased  
63 microbial mineralisation of <sup>14</sup>C-phenanthrene, or microbial activities, in planted soils when  
64 compared to corresponding unplanted controls. For this study, it was hypothesised that (i) both  
65 *M. sativa* and *S. bicolor* would show tolerance in PAH-diesel oil mixture contaminated soil,  
66 regardless of PAH concentration or diesel amendment; (ii) Increases in PAH mixture  
67 concentration, and diesel amendment, would decrease rates and extents of <sup>14</sup>C-phenanthrene  
68 mineralisation in soil; (iii) rates and extents of <sup>14</sup>C-phenanthrene mineralisation would be  
69 greater in planted treatments (monocultures or mixed cultures), and (iv) rates and extents of  
70 <sup>14</sup>C-phenanthrene mineralisation in treatments associated with mixed cultures would be  
71 greater than those of monocultures. To address these hypotheses, the following objectives  
72 were set: (i) to assess the tolerance (growth and survival) of *M. sativa* and *S. bicolor* in PAH-  
73 diesel oil mixture contaminated soil; (ii) to assess microbial mineralisation of <sup>14</sup>C-phenanthrene  
74 in soil spiked with a mixture of three PAHs and amended with diesel oil, and (iii) to evaluate  
75 and compare microbial mineralisation of <sup>14</sup>C-phenanthrene in PAH-diesel oil mixture in planted  
76 and unplanted treatments.

77

## 78 **2. Materials and Methods**

### 79 **2.1. Chemicals and other materials**

80 Non-labelled phenanthrene (>98%), benzo[a]pyrene (>97%), benzo[a]anthracene (>95%),  
81 sodium hydroxide (reagent grade), plate count agar (Fluka analytical), and toluene were  
82 purchased from Sigma-Aldrich, UK. [9-<sup>14</sup>C] phenanthrene (3.7 MBq/ml) was obtained from  
83 American Radiolabeled chemicals, Inc., USA, Goldstar liquid scintillation cocktail (LSC) from  
84 Meridian, UK, general purpose agar (agar-agar), general purpose grade Ringer's solution  
85 tablets, acetone (HPLC grade), as well as the chemicals used for preparing minimum basal  
86 salts (MBS) solution were acquired from Fisher Scientific, UK. Seeds of *M. sativa* and *S.*  
87 *bicolor* were purchased from Moles Seeds Ltd., UK and Chiltern Seeds, UK respectively.  
88 Commercial diesel was obtained from a local UK petrol station.

### 89 **2.2. Soil preparation**

90 A pristine agricultural soil was collected from a depth of 5 – 20 cm, from Myerscough  
91 Agricultural College, Preston, Lancashire, PR3 0RY, UK. The soil was a clay-loam (Dystric  
92 Cambisol) (FAO, 1988). Soil was air-dried and then passed through a 2 mm sieve. Thereafter,  
93 the sieved soil was stored in the dark at 4 °C until needed. Soil properties have been previously  
94 determined (Couling *et al.*, 2010) and are presented in Table SI 1. Air-dried soil was spiked  
95 with <sup>12</sup>C-PAH standard ( $\Sigma$ PAH = Phe + BaP + BaA) at 100 mg kg<sup>-1</sup> and 300 mg kg<sup>-1</sup>, as well  
96 as diesel (0.1% w/w) when applicable. Spiking was done using an inoculum approach  
97 following the protocol described by Doick *et al.* (2003). Briefly, the soil was rehydrated to  
98 approximately 35% moisture content with deionised water, after which 3 batches of 250 g soil  
99 were placed in a mixing bowl and spiked with <sup>12</sup>C-PAH standard in acetone:toluene (1:1, v/v)  
100 carrier solvent mixture. Solvent was allowed to disperse in soil and vented off in a fume  
101 cupboard. Soil was then thoroughly homogenised and distributed in pots according to the  
102 treatments described in Table 1.

### 103 **2.3. Plant-assisted biodegradation test**

### 104 **2.3.1. Assessment of seedling emergence and phytotoxicity**

105 Seedling emergence and growth test of both plant species was conducted following  
106 relevant OECD and USEPA guidelines (OECD., 2006; US EPA, 2012) with slight  
107 modifications. Prior to the growth test, a seed viability test was conducted using seeds ( $n =$   
108 10) of each species placed on a moistened filter paper in a petri dish. The petri dish was  
109 covered and placed in a controlled temperature room ( $21 \pm 1$  °C) and assessed daily for  
110 germination. The pot experiment was conducted in a glasshouse and had a completely  
111 randomised block design with three replicates. The specific treatments are described in Table  
112 1. Plastic pots (90 mm) were filled with 50 g soil, with a disc of filter paper fitted at the bottom  
113 to avoid soil loss. In addition, individual pot trays were fitted under each pot to control any  
114 leachate and avoid cross contamination. For monocultures, 10 seeds were sown into the pots,  
115 whereas 5 seeds each were sown for the mixed cultures (i.e. *M. sativa* + *S. bicolor*). .  
116 Germination, survival and general visual detrimental effects were assessed daily, while  
117 percentage seedling emergence and growth was determined after 21 d. Further, weekly  
118 measurements of plant heights were made while other visual toxic effects were also observed.  
119 At the end of the growth assay, planted treatments were destructively sampled in order to  
120 determine plant biomass. The shoots were harvested from the soil surface while the roots  
121 were carefully harvested after inverting the pots on a clean polythene sheet. Afterwards, roots  
122 were gently rinsed to detach soil from the surface and then dried with a clean paper towel.  
123 The fresh weights of the shoots and roots were measured, after which they were oven-dried  
124 at 60 °C for 24 h and their dry weights assessed. The root/shoot biomass ratios were then  
125 calculated.

### 126 **2.3.2. <sup>14</sup>C-Respirometry assay**

127 To assess plant-assisted biodegradation, evolution of <sup>14</sup>C-phenanthrene mineralisation in  
128 planted (after 0 and 21 d) and unplanted soils (after 0, 21, and 42 d) was monitored in 250 ml  
129 modified Schott bottles ( $n = 3$ ) at  $20 \pm 1$  °C, following the methods described by Reid et al.  
130 (2001). The biodegradation parameters assessed in this study include (i) the lag phase  
131 (defined as the time taken to reach 5% mineralisation); (ii) the fastest rate ( $\%^{14}\text{CO}_2 \text{ d}^{-1}$ ), and

132 (iii) the cumulative extent of mineralisation expressed as a percentage of the initial  $^{14}\text{C}$ -  
133 phenanthrene, which has been mineralised to  $^{14}\text{CO}_2$  during each sampling time.

### 134 **2.3.3. Enumeration of microbial cell numbers**

135 The number of indigenous microbes (total heterotrophs and PAH degraders) assessed as  
136 colony-forming units per grams soil dry weight (CFUs  $\text{g}^{-1}$ ) was estimated after 0 and 21 d  
137 (planted treatments), and 0, 21, and 42 d (unplanted treatments) following standard aseptic  
138 plate counting techniques (Lorch *et al.*, 1995). Cultures were grown (10 days,  $25 \pm 1$  °C).  
139 Microbial colonies were assessed after 4, 7 and 10 d. The ratio of degraders to total  
140 heterotrophs was also determined.

### 141 **2.3.4. Statistical analysis**

142 Data analysis was carried out using Sigmastat 13.0 (Systat Software, Inc.), and graphs  
143 were presented using SPSS Statistics (IBM Corp, version 24) and SigmaPlot for Windows  
144 13.0 (Systat Software, Inc.). The level of significance was at  $p < 0.05$ . Shapiro-Wilk Test - was  
145 used to determine normality of data whereas Levene's Test was used to determine equality of  
146 variance between groups. Statistical differences between groups were tested using one-way  
147 ANOVA ( $p < 0.05$ ). When  $p < 0.05$ , Tukey's Post Hoc was used to identify the locations of  
148 differences between groups. Where Levene's Test fails ( $p < 0.05$ ), Games Howell's Test which  
149 assumes variance non-equality was used for Post-Hoc analysis.

### 150 3. Results and Discussion

#### 151 3.1. Effects of PAHs and diesel on seedling emergence and growth

152 After 21 d incubation following sowing, no significant differences were observed regarding  
153 the percentage of emergence ( $p > 0.05$ ), even though values measured in soil spiked with 100  
154 mg kg<sup>-1</sup> ΣPAH were consistently greater than in soil amended with 300 mg kg<sup>-1</sup> ΣPAH (Table  
155 SI 2). Plant heights also followed this trend ( $p > 0.05$ ) in both mono- and mixed-cultures when  
156 compared to the control (Figure 1). Plant tolerance in PAH contaminated soils has been  
157 previously reported (Banks *et al.*, 2003; Cheema *et al.*, 2010; Hamdi *et al.*, 2012). For instance,  
158 the heights of *M. sativa*, *Brassica napus*, and *Lolium* sp. in pyrene amended soil were  
159 statistically similar to the uncontaminated controls which may imply species tolerance in the  
160 contaminated soil used (Ghanem *et al.*, 2010). However, PAHs in soil are generally not acutely  
161 toxic to plants (Chouychai *et al.*, 2007; Sverdrup *et al.*, 2007; Khan *et al.*, 2012). PAHs may  
162 be unavailable to interact with plants due to sorption in soil, a phenomenon which increases  
163 with increasing PAH hydrophobicity as well as soil organic matter content (Luthy *et al.*, 1997),  
164 and may thereby minimise PAH toxicity to plants in soil. Some studies however reported that  
165 diesel oil affected the germination and seedling emergence of plants and this effect has been  
166 attributed to volatile constituents of diesel fuel (Adam and Duncan, 2002; Bamgbose and  
167 Anderson, 2015). However, these plant growth effects are reduced significantly with ageing  
168 (Bona *et al.*, 2011; Wei *et al.*, 2017). Both *S. bicolor* and *M. sativa* tolerated the complex PAH-  
169 diesel oil mixtures contaminated soil as regards seedling emergence and plant growth under  
170 the prevalent assay conditions and no apparent signs of stress were observed. Such tolerance  
171 might be attributed to a combination of plant morphological and physiological characteristics,  
172 and soil-PAH interactions (Wenzel, 2009; Hamdi *et al.*, 2012; de Boer and Wagelmans, 2016).

#### 173 3.2. Effects of PAHs and diesel on plant biomass

174 A change in plant root biomass is also an important parameter that can be monitored during  
175 plant-enhanced biodegradation (Cheema *et al.*, 2010). High plant root biomass may favour  
176 microbial activity in soil through enrichment of rhizosphere (Banks *et al.*, 2003; Fan *et al.*,

177 2008; Wenzel, 2009). With respect to varying PAH concentrations and diesel amendment,  
178 variations in plant biomass among treatments were consistently observed in this study (Table  
179 2). Root biomass (dry weight) of *S. bicolor* across all treatments was greater than in the control.  
180 The greatest biomass value of *S. bicolor* was recorded in soil spiked with 100 mg kg<sup>-1</sup> ΣPAH  
181 and amended with diesel, as it exceeded the control by approximately 2 fold ( $p < 0.05$ ).  
182 However, *M. sativa* exhibited a significantly greater ( $p < 0.05$ ) biomass only in soil spiked with  
183 100 mg kg<sup>-1</sup> ΣPAH and amended with diesel, compared to the control. This putative hormetic  
184 response has been previously reported for *M. sativa*, where the plant roots were stimulated in  
185 soils contaminated with 1% and 1.5% of petroleum hydrocarbons, compared to controls (Kirk  
186 et al. 2002), including in corn in crude-oil contaminated soil (Salanitro et al. 1997). Observation  
187 of treatments with the same ΣPAH concentration (i.e. either 100 mg kg<sup>-1</sup> or 300 mg kg<sup>-1</sup>) in this  
188 study revealed that root biomass and root/shoot biomass ratio were generally greater in the  
189 diesel amended than unamended soils for both mono- and mixed-cultures (Table 2). For  
190 example, root biomass of *S. bicolor* and *M. sativa* monocultures in soil spiked with 100 mg kg<sup>-1</sup>  
191 ΣPAH and amended with diesel was greater by 33% and 31% respectively, compared to  
192 similar treatments without diesel amendment. In the same regard, root/shoot biomass ratios  
193 was greater by 40% and 18% in the 100 mg kg<sup>-1</sup> treatments with diesel for *S. bicolor* and *M.*  
194 *sativa* respectively, as well as by 28% and 45% in the 300 mg kg<sup>-1</sup> treatments with diesel.  
195 Generally, diesel amendment appeared to inhibit the adverse effects of PAHs on plant  
196 biomass and root/shoot biomass ratios; this was one of the key observations in this study. It  
197 is suggested that diesel in diesel-amended treatments may have promoted PAH partitioning  
198 into the diesel phase (Boyd and Sun, 1990), especially at low PAH concentrations in soil. This  
199 is such that closely-associating roots in the diesel-amended soil show minimal effects on  
200 biomass production compared to diesel-unamended treatments.

201 Overall, soil spiked with 100 mg kg<sup>-1</sup> and amended with diesel showed the greatest root  
202 biomass and root/shoot biomass ratios for both species within all treatments and growing  
203 patterns. With increase in ΣPAH concentration from 100 mg kg<sup>-1</sup> to 300 mg kg<sup>-1</sup>, root biomass  
204 and root/shoot biomass ratios generally decreased, especially for *M. sativa*; however, the

205 differences were not statistically significant ( $p > 0.05$ ). These results are similar to those  
206 presented by Cheema et al. (2010). The authors reported that after 65 d of plant growth, root  
207 biomass and root/shoot biomass ratio of *M. sativa* were mostly affected in soil amended with  
208 a mixture of 200 mg kg<sup>-1</sup> phenanthrene and 199.3 mg kg<sup>-1</sup> pyrene, when compared to rape  
209 seed exposed to the same treatment. These trends have also been observed for *Zea mays*  
210 L., *Lolium perenne* L. and *Trifolium repens*, exhibiting decreased biomass values with  
211 increasing concentrations of phenanthrene and pyrene mixtures in loam soil, but the  
212 differences were not statistically significant (Xu et al., 2006). These trends may have resulted  
213 from inherent non-acute toxicity of PAHs especially at higher concentrations in spiked soils  
214 (Wei et al., 2017). In addition, PAH-contaminated soils may inhibit flow of water and nutrients  
215 to plants, thereby affecting plant's ability to increase biomass especially at higher PAH  
216 concentrations (Reilley et al., 1996). The relationships between root and shoot biomass,  
217 especially root/shoot biomass ratios, are important indicators of plant health, although  
218 interpretation of such relationships is not always clear-cut (Mokany et al., 2006). Plant root  
219 systems utilise water and mineral nutrients from soil, and transports them to plant shoots,  
220 while shoot systems fix CO<sub>2</sub> needed for physiological purposes. It is thought that a reduced  
221 root/shoot biomass ratio is unfavourable for plants as it indicates shoot proliferation at the  
222 expense of root; however, reduced root/shoot biomass ratio especially at higher  
223 concentrations (300 mg kg<sup>-1</sup> ΣPAH) does not still exclude plant tolerance within the growth  
224 assay conditions (Harris, 1992; Cheema et al; 2010). One reason for the reduced root/shoot  
225 biomass ratios, especially at higher concentrations may have been due to increased root  
226 proliferation to allow increased transport of water and nutrients aboveground thereby  
227 increasing shoot biomass at the expense of root biomass, and hence a reduced root/shoot  
228 biomass ratio (Harris, 1992). This is evident in this present study where roots generally  
229 exhibited greater percentage decrease in biomass compared to shoots when ΣPAH  
230 concentration increased from 100 mg kg<sup>-1</sup> to 300 mg kg<sup>-1</sup>. For instance, percentage decreases  
231 in shoot biomass from 100 mg kg<sup>-1</sup> to 300 mg kg<sup>-1</sup> ΣPAH were approximately 4 % and 22 % in  
232 *S. bicolor* and *M. sativa*, respectively; whereas, the root biomass similarly decreased by

233 approximately 18 % and 25 %. This finding therefore implies that the rate at which root  
234 biomass proliferate may have been less compared to shoot biomass, which may have resulted  
235 in the reduced root/shoot biomass ratios observed at 300 mg kg<sup>-1</sup> ΣPAH compared to 100 mg  
236 kg<sup>-1</sup> ΣPAH. Similarly, roots are likely to be more susceptible to damage from soil contamination  
237 as they are in direct contact with soil, thereby adversely affecting water and mineral transport  
238 functions (Cheema *et al.*, 2010). As a result, greater energy may be expended on translocating  
239 carbohydrates produced above-ground to below-ground biomass resulting in an increased  
240 root/shoot biomass ratio (Harris, 1992; Reilley *et al.*, 1996). However, an evaluation of the  
241 moisture content of roots and shoots after harvesting both plant species did not present any  
242 significant difference ( $p > 0.05$ ) within each of the treatments, nor between each treatment  
243 and control (Figure SI 1). Hence, root functioning in terms of water transport may not have  
244 been significantly impaired due to PAH-diesel oil contamination in soil during the growth  
245 duration. These findings revealed reduced plant biomass and root/shoot biomass ratios for  
246 both plant species in PAH-diesel oil mixture contaminated soils especially in the 300 mg kg<sup>-1</sup>  
247 ΣPAH treatment, however potential toxicity or stress signs were not apparent throughout the  
248 growth period, which may support the notion of both plant species being tolerant of PAH-diesel  
249 oil contaminated soil.

### 250 **3.3. <sup>14</sup>C-phenanthrene mineralisation in unplanted and planted treatments**

251 The presence of a lag phase is indicative of the time needed to allow microbial adaptation  
252 in soil, and it has been suggested previously that a decreasing lag phase prior to mineralisation  
253 could be attributable to microbial adaptation processes (Macleod and Semple, 2002). Varying  
254 lag phases were observed in the unplanted soils, which significantly shortened ( $p < 0.05$ ) with  
255 in soil-contaminant contact time (Table 3). This was more pronounced in the planted soils  
256 (Table 3). Results revealed that the indigenous microorganisms in the unplanted control were  
257 catabolically active. However, microbial activities were much slower as revealed by longer lag  
258 phases, compared to the unplanted treatments (Table 3 and Figure 2A). The indigenous  
259 microorganisms in Myerscough soil may have access to various carbon sources, including

260 ubiquitously-distributed PAHs, although background PAH concentrations were considered to  
261 be negligible (Adebisi, 2010).

262 Across all unplanted treatments, the soil spiked with 100 mg kg<sup>-1</sup> ΣPAH generally exhibited  
263 shorter lag phases than those spiked with 300 mg kg<sup>-1</sup> ΣPAH with and without diesel  
264 amendment at 0 d. Overall, lag phases were not significantly different within and across all  
265 unplanted treatments and these ranged from 3.84 ± 0.50 d up to 5.34 ± 0.58 d at 0 d. Only  
266 treatments with 100 mg kg<sup>-1</sup> ΣPAH with and without diesel amendment presented lag phases  
267 significantly shorter ( $p < 0.05$  and  $p < 0.02$  respectively) when compared to untreated control  
268 soil. After 21 and 42 d, reduced lag phases, greater maximum rates and cumulative extents  
269 of mineralisation were observed in all treatments, compared to 0 d (Figures 2 - 3). Lag phases  
270 generally shortened to less than 1 d in both planted and unplanted treatments (Table 3).  
271 Rhodes *et al.*, (2008) also reported statistically shorter ( $p < 0.05$ ) lag phases after 42 and 84  
272 d soil-phenanthrene contact time in natural and artificial soils compared to those observed  
273 after 1 d contact time. An increase in indigenous microbial activities was observed in the  
274 planted (C3) compared to the unplanted (C4) controls (Table 3) as shown by significantly  
275 longer lag phases ( $p < 0.05$ ) and cumulative extents of <sup>14</sup>C-phenanthrene mineralisation ( $p <$   
276 0.0001). This shows the influence of both plant species at increasing indigenous microbial  
277 activities in soil, which may have implications for contaminant biodegradation. This was further  
278 reflected by the greater CFUs of total heterotrophs and PAH degraders in the planted controls  
279 than in the unplanted control, especially for *M. sativa* (Table 3). Plant roots release root  
280 exudates containing mineralisable oxygen, water, enzymes, and a diverse array of low  
281 molecular weight carbon-containing compounds such as amino acids, sugars, organic acids,  
282 and phenolics (Bais *et al.*, 2006). These root exudates may enrich the rhizosphere and serve  
283 as readily-mineralisable carbon sources for microorganisms involved in symbiotic root-  
284 microbe interactions (Bais *et al.*, 2006; Wenzel, 2009). Continuous mineralisation and  
285 incorporation of these carbon sources increases microbial biomass, thereby supporting  
286 microbial growth, activity, and contaminant biodegradation (Guo *et al.*, 2017). Such symbiotic  
287 root-microbe interactions in soil have been previously reported for *M. sativa* (Fan *et al.*, 2008)

288 and *S. bicolor* (Banks *et al.*, 2003; Muratova *et al.*, 2009a). Specifically, enzymatic metabolites  
289 via cationic peroxidases from *M. sativa* and *S. bicolor* are key mechanisms for PAH  
290 biodegradation in soil in the presence of the plant species (Dubrovskaya *et al.*, 2017).

291 Mineralisation followed immediately after each lag phase period. At 0 d, fastest rates ( $0.98$   
292  $\pm 0.37$  %  $^{14}\text{CO}_2 \text{ d}^{-1}$ ) and greatest cumulative extents of  $^{14}\text{C}$ -phenanthrene mineralisation ( $59.27$   
293  $\pm 6.09$  %) were observed only in the unplanted treatment with  $100 \text{ mg kg}^{-1}$   $\Sigma\text{PAH}$  and amended  
294 with diesel ( $p < 0.05$ ). The corresponding  $300 \text{ mg kg}^{-1}$   $\Sigma\text{PAH}$  treatment exhibited the slowest  
295 rates ( $0.20 \pm 0.002$  %  $^{14}\text{CO}_2 \text{ d}^{-1}$ ) as well as the lowest cumulative extents ( $24.68 \pm 3.48$  %) of  
296 mineralisation. This trend was further reflected by a greater ratio of degraders to total  
297 heterotrophs in soil with  $100 \text{ mg kg}^{-1}$   $\Sigma\text{PAH}$ , compared to soil with  $300 \text{ mg kg}^{-1}$   $\Sigma\text{PAH}$  as shown  
298 in Figure SI 2A. However, microbial numbers (PAH degraders or total heterotrophs) within and  
299 across corresponding treatments were not significantly different ( $p \geq 0.05$ ) (Table 3). In the  
300 unplanted treatments at 21 d (Table 3), rates of mineralisation were fastest ( $p < 0.0001$ ) in soil  
301 spiked with  $300 \text{ mg kg}^{-1}$   $\Sigma\text{PAH}$  especially the diesel unamended treatment; whereas,  
302 cumulative extents of mineralisation were greatest in soil with the  $100 \text{ mg kg}^{-1}$   $\Sigma\text{PAH}$  without  
303 diesel. The maximum rates of mineralisation within the planted treatments in comparison to  
304 their corresponding unplanted controls were statistically similar ( $p > 0.05$ ). This observation is  
305 consistent with previous findings where microbial respiration was not affected by plant species  
306 identity (Oyelami *et al.*, 2013), and have been suggested to be due to spatial limitations  
307 between indigenous microorganisms and plants in soil. Considering biodegradation  
308 parameters such as lag phases, fastest rates and cumulative extents of  $^{14}\text{C}$ -phenanthrene  
309 mineralisation, observations at 0 d appeared to depict mineralisation patterns which may have  
310 been largely influenced by the concentration of freshly spiked  $\Sigma\text{PAH}$  in soil. It is well known  
311 that freshly spiked PAHs are more mobile and bioavailable in soil than aged PAHs (Semple *et*  
312 *al.*, 2007), due to minimal influence of soil-contaminant sequestration processes (Luthy *et al.*,  
313 1997). Sorption forces are usually more apparent at lower concentrations (Pignatello and Xing,  
314 1996), hence, soil with higher concentrations of freshly spiked PAHs may be subject to greater  
315 contaminant bioavailability compared to soil with lower concentrations (Hwang and Cutright,

316 2004b, a). Since PAHs are potentially toxic, adverse effects on soil enzymatic, as well as  
317 microbial numbers and catabolic activities are likely to be observed (Kanaly and Harayama,  
318 2000). In this present study, PAH inherent toxicity to indigenous microorganisms, especially  
319 in soils spiked with 300 mg kg<sup>-1</sup> ΣPAH, may have resulted in the pattern observed of  
320 biodegradation parameters in unplanted soil at 0 d soil-PAH contact time. This result is  
321 consistent with those of Couling et al. (2010) who reported greater biodegradation parameters  
322 in soil spiked with lower concentrations of individual PAHs, and/or a mixture of naphthalene,  
323 phenanthrene and pyrene, with single or multiple dosing of each concentrations. However, the  
324 differences between biodegradation parameters at low and high PAH concentrations were  
325 usually statistically similar ( $p > 0.05$ ) (Couling *et al.*, 2010). In addition, while Oyelami et al.  
326 (2013) observed that unplanted soils amended with different concentrations of PAH mixtures  
327 showed corresponding responses in degrader numbers and activities which may have  
328 resulted in consequent <sup>14</sup>C-phenanthrene mineralisation, observations from this present study  
329 did not generally show such trends (Table 3 and Figure SI 2 - SI 3).

330 The rates of PAH mineralisation in planted and unplanted treatments were generally  
331 statistically similar; however, cumulative extents of mineralisation also need to be considered  
332 to evaluate plant-assisted biodegradation. Cumulative extents of mineralisation at 21 d were  
333 significantly greater in soils spiked with 300 mg kg<sup>-1</sup> ΣPAH with diesel for both *S. bicolor* ( $p <$   
334  $0.0001$ ) and *M. sativa* ( $p = 0.003$ ) monocultures, compared to corresponding unplanted  
335 treatments. However, a contrasting trend was generally observed ( $p < 0.05$ ) in soils spiked  
336 with 100 mg kg<sup>-1</sup> and 300 mg kg<sup>-1</sup> ΣPAH without diesel, which implied that plant-assisted  
337 biodegradation in these diesel-unamended treatments was not evident in these soils. Similar  
338 findings has also been reported previously (Smith *et al.*, 2011; Cennerazzo *et al.*, 2017). For  
339 instance, Cennerazzo *et al.* (2017) reported that biodegradation in soil spiked with 300 mg kg<sup>-1</sup>  
340 <sup>1</sup> phenanthrene within a 21 d *Lolium perenne* monoculture was not significantly different from  
341 the unplanted treatment. In contrast, cumulative extents of mineralisation in soil spiked with  
342 100 mg kg<sup>-1</sup> ΣPAH with diesel from only *M. sativa* monoculture were significantly greater ( $p =$   
343  $0.013$ ) than that in corresponding unplanted treatment. Cumulative extents of mineralisation

344 were generally statistically similar ( $p > 0.05$ ) within planted treatments (mono- and mixed  
345 cultures). The only exception was in *S. bicolor* planted soil spiked with 300 mg kg<sup>-1</sup> ΣPAH and  
346 without diesel, which showed a significantly greater ( $p = 0.003$ ) cumulative extents of  
347 mineralisation compared to corresponding *M. sativa* treatment. Further, cumulative extents of  
348 mineralisation within treatments were statistically similar ( $p > 0.05$ ) at 42 d, except for soil  
349 spiked with 100 mg kg<sup>-1</sup> ΣPAH without diesel where cumulative extents of mineralisation were  
350 significantly greater ( $p < 0.05$ ) than corresponding treatment with diesel.

351 In this study, diesel amendment generally inhibited the rates and cumulative extents of <sup>14</sup>C-  
352 phenanthrene mineralisation in soils at 21 d and 42 d; however, the trend was not consistent,  
353 as had been previously documented for other NAPLs (Lee *et al.*, 2003). Diesel, itself being a  
354 hydrophobic non-aqueous phase liquid (Adam and Duncan, 1999), contains the greatest  
355 amount of PAHs and aromatics when compared to other medium distillate fuel oils (Wang *et al.*  
356 *et al.*, 1990). It is therefore suggested that due to its hydrophobic nature, diesel may further  
357 increase PAH partitioning processes (Boyd and Sun, 1990), especially in soils with low  
358 concentrations of PAHs. Hence, decreased PAH mobility, bioavailability, toxicity, and  
359 biodegradation may occur, as also evident from the results of plant biomass and root/shoot  
360 biomass ratios previously discussed. Therefore, soil with greater PAH concentrations and  
361 amended with diesel may show greater rates and extents of mineralisation compared to one  
362 with lower PAH concentrations, especially in the presence of relevant plant species. In  
363 addition, rates and extents of mineralisation are likely to be greater in diesel unamended  
364 treatments and especially at lower PAH concentrations since an additional sorbent phase  
365 (diesel) is absent. The modifying effects of diesel amendment on rates and extents of PAH  
366 mineralisation in spiked soil may be dependent on concentration of diesel amended (Alejandra  
367 *et al.*, 2014), and these effects are likely to be greater in highly weathered field-contaminated  
368 soils (Wei *et al.*, 2017). In another study, phenanthrene degradation was reported to have  
369 increased in a pasture soil with diesel concentration of 0 - 2,000 mg kg<sup>-1</sup>, but then decreased  
370 when diesel concentration was increased to 20,000 mg kg<sup>-1</sup> (Swindell and Reid, 2006). Towell  
371 *et al.* (2011) also investigated the effect of cable oil concentration on biodegradation of <sup>14</sup>C-

372 phenyldodecane in an agricultural soil and reported that even though microbial respiration  
373 increased with increasing oil concentration (0.001 - 10 %, w/w dry weight of soil),  
374 mineralisation of <sup>14</sup>C-phenyldodecane decreased. In this present study, greater rates and  
375 cumulative extents of mineralisation at 21 and 42 d were mostly observed in diesel  
376 unamended treatments with similar ΣPAH concentrations (100 mg kg<sup>-1</sup> or 300 mg kg<sup>-1</sup>). The  
377 nature of NAPLs and associated concentration are factors to be considered in PAH  
378 biodegradation. Key questions to answer in future investigations are, at what concentration  
379 and soil-contaminant contact time does diesel oil increase or decrease PAH biodegradation,  
380 as well as identifying the mechanisms controlling the influence of diesel oil on PAH  
381 bioavailability in aged soil? Such investigations may have implications for biodegradation of  
382 complex PAH-diesel oil mixtures, especially in historically contaminated soils.

383 Based on previous studies (Xu *et al.*, 2006; Meng *et al.*, 2011), it was expected that a mixed  
384 culture of both plant species used in this study would co-enhance rates and extents of <sup>14</sup>C-  
385 phenanthrene mineralisation in soil compared to their individual monocultures, rather, the  
386 mixed culture associated treatments did not significantly enhance rates and extents of <sup>14</sup>C-  
387 phenanthrene mineralisation (Table 3). Either of the monocultures generally exhibited  
388 significantly greater ( $p < 0.05$ ) extents of mineralisation compared to the mixed culture.  
389 Oyelami *et al.* (2013) also reported that plant species richness had no significant effects on  
390 phenanthrene biodegradation in long-term aged soil. To the best of our knowledge, there have  
391 been no published studies evaluating the plant-assisted biodegradation potential of *M. sativa*  
392 and *S. bicolor* mixed cultures in PAH-diesel oil contaminated soil. Belowground interactions  
393 between many plant roots are yet to be understood and fully investigated (Bais *et al.*, 2006).  
394 Although based on daily visual assessment, plant growth aboveground in the controls did not  
395 appear limited, however, plant biomass and root/shoot biomass ratios were generally more  
396 reduced in mixed cultures than individual monocultures both within control and PAH-diesel oil  
397 amended treatments. An antagonistic interaction between the roots of both plant species in  
398 this study may not be totally excluded (Hedge and Miller, 1990; Muratova *et al.*, 2009a); this  
399 is subject to further investigations. In this regard, it is speculated that greater energy may have

400 been expended by both plant roots towards surviving competition and associated adverse  
401 effects, rather than supporting microbial activity in the mixed culture as generally shown in  
402 Table 3. Similarly, associated microorganisms within the rhizosphere may also expend energy  
403 competing for preferable rhizospheric microhabitats rather than co-enhance biodegradation  
404 (vanVeen *et al.*, 1997). Such counter-productive survival interactions within the rhizosphere  
405 may affect the combined potential of both plant roots as well as associated microorganisms to  
406 better enhance rates and extents of <sup>14</sup>C-phenanthrene mineralisation in the PAH-diesel oil co-  
407 contaminated soil. Whether plant-assisted biodegradation of PAHs in soil, within a mixed  
408 culture of both plant species, will be observed during an extended growth period is subject to  
409 further investigations.

#### 410 **4. Conclusion**

411 *S. bicolor* and *M. sativa* mono- and mixed- cultures were tolerant of the PAH-diesel oil  
412 amended soil. Plant-assisted biodegradation of PAH-diesel oil mixtures in soil, within the  
413 growth duration examined, was greater in *S. bicolor* or *M. sativa* monocultures compared to  
414 the mixed culture of both plant species. Overall, increase in PAH concentration reduced plant  
415 biomass and root/shoot biomass ratios, as well as adversely affected lag phases, and rates  
416 and extents of <sup>14</sup>C-phenanthrene mineralisation especially at initial stages of soil-contaminant  
417 contact. In contrast, maximum rates and cumulative extents of <sup>14</sup>C-phenanthrene  
418 mineralisation were greater at advanced stages of soil-contaminant contact time especially in  
419 the more concentrated PAH-contaminated soils with monocultures of the plant species used.  
420 Diesel amendment supported plant biomass production as well as increase in root/shoot  
421 biomass ratios, however, appeared to inhibit rates and extents of <sup>14</sup>C-phenanthrene  
422 mineralisation in soil. The mechanisms through which diesel oil controls the fate and behaviour  
423 of complex PAH mixtures in soil should be further investigated. These may have implications  
424 for the risk assessment and remediation of PAHs in soil.

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430 **6. Supplementary Information**

431 Supplementary data on “Mineralisation of <sup>14</sup>C-phenanthrene in PAH-diesel contaminated  
432 soil: Impact of *Sorghum bicolor* and *Medicago sativa* mono- or mixed culture” are available.

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Figure 1. Weekly plant heights across all treatments ( $p > 0.05$ ).

Figure 2. Development of  $^{14}\text{C}$ -phenanthrene mineralisation at 0 d (A), 21 d (B) and 42 d (C) respectively. Control, C4 (■); C5 (▼), and C6 (Δ); C7 (●); C8 (○). Note the different scale on y-axis.

Figure 3. Development of  $^{14}\text{C}$ -phenanthrene mineralisation at 21 d in monocultures of *S. bicolor* (A) and *M. sativa* (B), and mixed culture (C) respectively. Control = C3 (■); T1 (▼); T2 (Δ); T3 (●); T4 (○). Note the different scale on y-axis.







