ENHANCEMENT OF ^{14}C-PHENANTHRENE MINERALISATION WITH ROOT BIOMASS FROM PAH-NAPL CONTAMINATED SOIL

Gabriela M Vázquez-Cuevas, Carly J Stevens and Kirk T Semple*

Lancaster Environment Centre, Lancaster University, Lancaster, LA1 4YQ, UK

*Corresponding author: Phone no. +44 (0)1524 510554; email: k.semple@lancaster.ac.uk

Abstract

The study of the beneficial effects of the interaction between plants and soil microorganisms towards bioremediation of contaminated soil has been studied over the past 30 years. This subject has been summarized as the process where organic contaminants can be removed from the soil through the interaction between roots and catabolic microbial populations. This study assessed the tolerance of different plant species against polycyclic aromatic hydrocarbon-non aqueous phase liquid (PAH-NAPL) contaminated soil and the feasibility of the use of their root biomass to promote the biodegradation of ^{14}C-phenanthene. Toxicity results showed that seeds germination was not affected by the presence of PAHs. Furthermore, mineralisation of ^{14}C-phenanthrene was significantly enhanced by the addition of root biomass after at least two weeks incubation. Moreover, bacterial numbers did not show a significant relationship with ^{14}C-phenanthrene mineralisation. Results showed that the higher mineralisation of ^{14}C-phenanthrene is not related to an increase on the microbial numbers as is normally assumed.

Keywords: Plant-enhanced, mineralisation, PAHs, diesel, roots, NAPL

1. Introduction

Soil bioremediation can be promoted by different biological mechanisms through the action of microorganisms, soil invertebrates and plants. Where plants are involved, this is commonly known as phytoremediation or plant-assisted remediation, which has been defined as the use of higher plants to treat or stabilise contaminated soil (Wenzel, 2009). A key partnership in the removal of pollutants from soil involves the impact of plants and plant roots on the enhancement of microbial degradation in soil (Anderson et al., 1993). Despite of this, the mechanisms in which plants stimulate microbial degradation is not fully understood (Anderson et al., 1993; Pilon-Smits, 2005). The specific mechanisms of the biodegradative processes are likely to vary between plant, microbial species and pollutant type due to a number of physico-chemical and environmental factors (Pilon-Smits, 2005).

Several plant species have been tested to examine their potential for phytoremediation of PAHs under specific soil conditions (Muratova et al., 2008; Rezek et al., 2008). So far, grasses such as fescue (*Festuca* sp.) and ryegrass (*Lolium* sp.), along with legumes (e.g. *M. sativa*) have shown to be suitable options to clean up hydrocarbons from the soil. These two functional groups are often selected due to their extensive fibrous root structure, which increases the soil-rhizosphere contact surface and penetration capacity (Aprill and Sims, 1990; Pilon-Smits, 2005), and their role on the fixation of nitrogen from the atmosphere into the soil (Wenzel, 2009), respectively. Moreover, it also needs to take into consideration that these species should be able to germinate and grow in highly contaminated soil, and in most cases under nutrient limiting conditions (Moubasher et al., 2015; Wenzel, 2009). It is generally agreed that a preliminary and effective way to test the phytoremediation potential of specific plant species is through phytotoxicity tests (Muratova et al., 2008; Trapp and Karlson, 2001). These types of assays use germination and early growth assessments as endpoints to determine suitability for growth in contaminated soil. For remediation of PAHs,

69 phytotoxicity assays have become a useful tool to assess plant tolerance to specific soil
70 conditions (Baek et al., 2004; Rorison and Robinson, 1986).

71 Phytotoxicity assays are usually done alongside with the quantification of initial and final
72 concentrations of the targeted pollutant (e.g. Aprill and Sims (1990); Corgié et al. (2003);
73 Günther et al. (1996); Joner et al. (2001); Miya and Firestone (2001); Olson et al. (2007);
74 Rezek et al. (2008); Smith et al. (2011); Thompson et al. (2008) for either single or mixtures
75 of PAHs). Furthermore, other approaches to test the effect of plants on the removal of PAHs
76 from soil have not been explored as extensively as the initial/final concentration assessment.
77 Only a small number of studies have been reported where ^{14}C-labelled pollutants were used
78 to test the potential of plant-associated microbial communities to mineralize hydrocarbons.
79 This approach offers a more realistic and accurate assessment of the fate and behaviour of a
80 specific pollutant, avoiding taking into account other sources of carbon being simultaneously
81 used by the soil microorganisms.

82 Using this approach, the impact of the root system, root biomass and root-derived substrates
83 towards has been assessed For example, Yoshitomi and Shann (2001) used exudates from
84 corn (*Zea mays* L.) to look at the mineralisation of ^{14}C-pyrene in rhizospheric and root-
85 amended soil over 80 d; they found no significant difference between these two conditions,
86 suggesting that the catabolic enhancing factor was the root exudate and not the whole root
87 system. Mueller and Shann (2007) studied the ^{14}C-pyrene mineralisation in soil amended
88 with red maple root extracts and the influence of inorganic nutrient addition. In this case,
89 rates of mineralisation were greater when rhizosphere soil was used; however, these were
90 inhibited by inorganic nutrient amendments. Moreover, Oyelami et al. (2013) studied not
91 only the effect of plant species in the mineralisation of ^{14}C-phenanthrene, but they also
92 looked at how species diversity, composition and soil fertility could enhance the degradation
93 of ^{14}C-phenanthrene in soil. The authors found that one of the most important elements was

the soil fertility, leaving the plant type and composition as secondary factors (Oyelami et al., 2013).

The hypothesis for this investigation was that the amendment of root biomass paste simulating root decay and turnover in PAH-NAPL contaminated soil enhances the mineralisation of ^{14}C-phenanthrene. To address this, the aims were (1) to assess the tolerance of different plant species against PAH-NAPL highly contaminated soil, and (2) to use the roots from the most resistant species as soil amendment to biostimulate the biodegradation of ^{14}C-phenanthrene, assessed through its mineralisation. Results from this study will allow assessing in a more accurate and controlled manner any possible PAH underestimations often misinterpreted as pollutant dissipation. Additionally, findings will contribute to the understanding of the influence of root decay and turnover on the catabolic response of microbial communities in PAH-NAPL contaminated soil.

2. Material and methods

2.1 Phytotoxicity testing

2.1.1 Soil spiking procedure

A clay loam soil with an organic matter content of 2.7 % was collected from Myerscough, Preston, U.K., a complete characterisation is described elsewhere (Towell et al., 2011). Following wet sieving (25% moisture content) through a 2 mm mesh, soil was spiked with a molecularly balanced mixture of phenanthrene (Phe), benzo(a)anthracene (B[a]A), and benzo(a)pyrene (B[a]P) to deliver final total concentrations of 100 and 300 mg kg^{-1}, and 0.1% (w/w) diesel using acetone:toluene (1:1) as a carrier. In addition, activated charcoal (0.1 %) was also included as a variable given its ubiquity in the environment and significant impact on the fate and behaviour of contaminants in soil (Rhodes et al., 2008), The selected amount of activated charcoal has been previously reported to be high enough to significantly reduce the bioaccessibility of 14C-phenanthrene (Rhodes et al., 2008). Soil preparation and spiking procedure followed the methods described by Doick et al. (2003) for the introduction of PAHs into wet soil using a stainless-steel spoon and a soil inoculum approach.

2.1.2 Seeds emergence and early growth

The seeds emergence and early growth studies included 9 plant species (Table 1) using 8 different soil treatments (n = 3) and 4 controls (Table 2) with a total of 324 experimental units in a completely randomised arrangement. Methodology followed the guidelines 208 (OECD, 2003) and OCSPP 850.4100 (US EPA, 2012), both focused on the study of ecological effects of chemicals in the environment. Given that these guidelines were originally developed for pesticide testing, minor modifications were made based on specific phytoremediation assays (Banks et al., 2003; Gao and Zhu, 2004; Muratova et al., 2009; Phillips et al., 2006; Smith et al., 2006). In short, 50 g of spiked soil was placed into 9 cm diameter pot with a disc of filter paper at the bottom of each to prevent soil loss from the

draining holes. Further, petri dishes were placed under each pot to control any leachate and prevent cross-contamination. In each pot, 10 seeds of the appropriate species (Table 1) were sown at equal distances from each other on soil surface. The pots were placed in a glasshouse to complete a 4-week growing period at ~25ºC with a 16/8 h photoperiod. At an initial stage, pots were covered with a petri dish to promote germination and watered daily from the top to prevent seed dryness, recording the daily germination rate. Once 50% of the control seeds of each species emerged, the seeds emergence test was considered finished, and the early growth assessment started. As the different plant species had different germination times, pots from each species was considered to be part of an independent experiment, and treatments were only compared against their counterpart from the same species. The endpoints of the growth test were assessed weekly over a period of 21 d after 50% emergence of the control pots as survival. During the growth test, plants were watered from the bottom. At the end of the assay shoot and root biomass was harvested and oven dried for 72 h at 60ºC (Langer et al., 2010).

2.2 Rhizo-enhanced ^{14}C-phenanthrene mineralisation in soil

2.2.1 Root amendments and soil preparation

Based on the results from the germination and phytotoxicity test (Section 2.1), four of the species were identified as resistant to the PAH-NAPL mixture due to none or minimal detrimental effects compared to control treatments at any of the PAHs concentrations. Seeds of these species; two grasses (*Sorghum bicolor* L., *Lolium perenne* L.) and two legumes (*Glycine max* L. and *Medicago sativa* L.), were sown into pristine soil from the same location as the one used for the phytotoxicity stage. Seeds were grown in a glasshouse under standard growth conditions (US EPA, 2012) for 21 d after seed emergence. After the growth period, roots were harvested and cleaned by three consecutive washes with sterile deionized water (Miya and Firestone, 2001). Each wash consisted on shaking the root into 100 ml sterile

156 deionized water for 30 minutes on an orbital shaker (Muratova et al., 2003) at 70 rpm.
157 Finally, the clean roots were blotted dry, cut into 1-2 mm pieces, and ground. The ground
158 root tissue was mixed with a minimum amount of distilled water to facilitate homogeneity
159 (Mueller and Shann, 2007) and then incorporated into the ^{12}C spiked soil according to the
160 treatments shown in Table 3 to get a final root amendment content of 0.5% (dry weight
161 based). Additionally, control soil without ^{12}C PAHs were also included, these had as a main
162 objective to allow a clear visualisation of how the absence of PAHs affect the mineralisation
163 process under the different tested conditions.

164 2.2.2 ^{14}C-Phenanthrene mineralisation in soil

165 The ^{12}C-PAHs spiked and root amended soil was stored in amber jars at 21 ± 1 ºC in the dark
166 over 28 d to simulate ageing. To test how the root amendment affected ^{14}C–phenanthrene
167 mineralisation rates over the time, three contact times were selected to conduct a
168 respirometric assay following the method described by Doick and Semple (2003) and Semple
169 et al. (2006). At 1, 14 and 28 d after ^{12}C spiking and root amendment, 10 g (dry wt) incubated
170 soil from each treatment (Table 3) was placed into 250 ml modified Teflon lined screw cap-
171 Schott bottles (respirometers) in triplicate. Each respirometer also contained 30 ml of
172 sterilized minimal basal salts (MBS) medium (Fenlon et al., 2007) to give a soil:liquid ratio
173 of 1:3 (Doick and Semple, 2003), and incorporated a suspended 7 ml glass scintillation vial
174 containing 1 ml 1 M NaOH solution which served as a $^{14}CO_2$ trap. At every time point, each
175 respirometer was spiked with $^{12/14}C$-phenanthrene (10 mg kg $^{-1}$ / 56 Bq g $^{-1}$, dry wt).
176 Respirometers were incubated at 21 ± 1 ºC in the dark onto an orbital shaker at 100 rpm for
177 14 d. The ^{14}C-activity in the $^{14}CO_2$ traps was assessed every 24 h by replacing the vial
178 containing NaOH and adding 5 ml Goldstar liquid scintillation cocktail. After storage in the
179 dark for 24 h, the ^{14}C-activity was quantified using a Canberra Packard Tricarb 2250CA
180 liquid scintillation analyser. An analytical blank (pristine soil without ^{14}C-phenanthrene) was

also set up to establish the level of background activity. The endpoints of this assay were the lag phase (defined as the period of time before mineralisation reached 5%), maximum rate (determined by the amount of $^{14}CO_2$ produced at any 24 h period of time), and the maximum extent of mineralisation after 14 d (Fenlon et al., 2007).

2.2.3 Microbial enumeration

Total heterotrophic and PAH degrading bacteria for each treatment was quantified by assessing the number of colony forming units (CFUs) following standard serial dilutions. Plates were prepared using tryptone glucose yeast agar, also known as plate count agar (PCA), and general purpose agar (GPA) amended with minimal basal salts (NaCl, $(NH_4)_2SO_4$, KNO_3, KH_2PO_4, K_2HPO_4, $MgSO_4 \cdot 7H_2O$), trace elements ($LiCl(LiBO_2)$, $CaSO_4 \cdot 5H_2O$, $ZnSO_4 \cdot 7H_2O$, $Al_2(SO_4)_3 \cdot 16H_2O$ $NiCl \cdot 6H_2O$, $CoSO_4 \cdot 7H_2O(CoNO_3)$, KBr, KI, $MnCl_2 \cdot 4H_2O$, $SnCl_2 \cdot 2H_2O$ and $FeSO_4 \cdot 7H_2O$) and 0.2 % phenanthrene as sole source of carbon (Fenlon et al., 2007). Microbial enumeration was incubated at 21 ± 1 ºC and assessed before and after the mineralisation experiment as recommended by Oyelami et al. (2013).

2.3 Statistical analysis

Statistical analysis was done using IBM SPSS Statistics software version 21. In all cases, normality of the residuals was verified using the Shapiro-Wilk test with a 95% confidence interval. In cases where data did not show a normal distribution, different data transformations were tested before using a non-parametric test. Data with normal distribution was analysed using an ANOVA analysis of variance and Tukey HSD post hoc test. When data was not normally distributed and no transformation was possible, Kruskal-Wallis mean rank of the group's analysis was used. Paired *t*-tests were used to test differences between time points.

204 3. Results

205 3.1 Phytotoxicity test

206 3.1.1 Seeds emergence and early growth as survival

207 The impacts of the chemicals on seeds emergence were investigated (Figure 1). It was found
208 that the germination of *F. rubra*, *C. angustifolium* and *P. lanceolata* in the growth control
209 (C_g) was less than 50%. Therefore, these have not been included in any further analysis.
210 Overall, the number of seeds that germinated after 7 d was not affected by the presence of
211 PAHs or the other amendments. Only *T. repens* displayed significant differences across the
212 different treatments ($F = 3.546$, $p = 0.005$), presenting a significantly lower germination rate
213 in soil without (T1, $p = 0.040$) and with (C_d, $p = 0.026$) diesel (Figure 1).

214 None of the species showed significant differences in survival within the different treatments
215 ($p > 0.05$). Nonetheless, the lowest survival at the end of the experiment was presented by *T.*
216 *repens* and *A. millefolium* when sown in the diesel control (C_d), suggesting the sensitivity of
217 these two species towards diesel. Moreover, *S. bicolor* displayed the highest overall survival,
218 being the only species to reach 100% survival.

219 3.1.2 Biomass

220 For both above and belowground biomass (dry wt), out of the six species tested, only *M.*
221 *sativa* and *G. max* were not significantly affected ($p > 0.05$) by any of the treatments (SI 1).
222 Furthermore, shoot and root biomass of *L. perenne*, *S. bicolor* and *T. repens* showed
223 significant differences across the soil conditions. *L. perenne* had significantly higher shoot
224 biomass when PAHs were present in the soil ($F = 3.3776$, $p = 0.003$), while only one
225 treatment (T1) significantly enhanced root biomass ($F = 6.534$, $p < 0.001$). *S. bicolor*
226 aboveground biomass was significantly reduced by the presence of PAHs ($F = 5.663$, $p <$
227 0.001), while the root biomass was promoted when grown on contaminated soil ($F = 2.758$, p
228 $= 0.019$). *A. millefolium* only showed significant differences regarding root biomass,

displaying a significant reduction ($F = 5.250$, $p = 0.001$) on treatments with the higher PAH concentrations.

Root/shoot ratio, was affected in three of the six species tested (SI 2). *L. perenne* showed an increased ratio in the treatment containing 100 mg kg^{-1} and diesel (T1) and only diesel (C_d) when compared to the growth control ($F = 5.999$, $p < 0.001$). Similarly, *S. bicolor* ($F = 3.395$, $p = 0.006$) and *T. repens* ($F = 2.766$, $p = 0.019$) also presented significantly higher ratios in the presence of contaminants.

3.2 Mineralisation of ^{14}C-phenanthrene in soil

The mineralisation of ^{14}C-phenanthrene was measured in soils under different conditions (Figures 2 – 5 and SI 3). For the treatment in which soil was spiked with the mixture of PAHs only (Treatment A, Figure 2), there were significand differences at each time point. Nonetheless, specific effects were different throughout the incubation time: one day after spiking and root amendment (Figure 2a), *L. perenne* amended soil showed a significantly shorter lag phase than the rest of the treatments ($F = 13.851$, $p = 0.001$), while significant higher total extents ($F = 19.473$, $p < 0.001$) and maximum rates of mineralisation ($F = 5.531$, $p = 0.016$) were observed in soil without root amendments. After 14 d of incubation (Figure 2b), lag phases were significantly faster ($F = 39.337$, $p < 0.001$) and total extents higher ($F = 42.555$, $p < 0.001$) in the soils containing root amendments. In the soils incubated for 28 d, trends observed after 14 d continued; the lag phase ($F = 49.468$, $p < 0.001$) was significantly shorter in the *L. perenne* amendment compared to the non-amended treatment (Figure 2c). Maximum rates ($F = 69.700$, $p < 0.001$) and total extents of mineralisation ($F = 85.097$, $p < 0.001$) were also significantly higher in all root-amended treatments compared to the non-amended soil.

When 0.1% (dry wt) activated charcoal (Treatment B) was added to the PAH mixture, statistically significant differences were observed at each of the three sampling times (Figure

3). One day after spiking, *S. bicolor* amendment had a negative effect on the ^{14}C-phenanthrene mineralisation; this is reflected in a longer lag phase than the rest of the treatments ($F = 3.973$, $p = 0.004$). Additionally, although the maximum rates of mineralisation were similar across treatments ($F = 2.960$, $p = 0.090$), total extents of the treatment amended with *G. max* and the rootless soil were significantly higher ($F = 15.653$, $p = 0.001$) than the grass-amended treatments (*L. perenne* and *S. bicolor*). After 14 d of incubation (Figure 3b), lag phases were longer in the absence of roots compared to the root-amended treatments ($F = 12.586$, $p = 0.003$). At this time point, significant increases in the maximum rates of mineralisation ($F = 8.863$, $p = 0.008$) were observed when soil was amended with *G. max* roots. The total extents of mineralisation also significantly increased in the presence of roots from all of the species ($F = 124.072$, $p < 0.001$). After 28 d of incubation (Figure 3c), significant differences were found for the maximum rates and total extents of mineralisation ($p < 0.05$). The maximum rates in soils containing *L. perenne* were significantly higher than in the other treatments ($F = 25.505$, $p < 0.001$), while total extents were enhanced in the presence of roots from any species compared to the non-amended soil in all the cases ($F = 65.824$, $p < 0.001$).

To observe the effect that a NAPL can have on the mineralisation of ^{14}C-phenanthrene (Treatment C), 0.1% (w/w) diesel was added to the PAH mixture (Figure 4). By the first and second sample times of this treatment (Figure 4a-b), lag phases were significantly shorter in the treatments amended with grass roots after 1 day ($F = 23.017$, $p = 0.002$), and on non-amended soil after 14 d ($F = 6.832$, $p = 0.011$). In contrast, maximum rates and total extents of mineralisation remained unaffected in the other treatments ($p > 0.05$). By the end of the 28 d incubation (Figure 4c), the lag phases were significantly shorter in the *L. perenne* condition ($F = 12.199$, $p = 0.003$) compared to the rest of the treatments. Both maximum rates ($F = 24.143$, $p < 0.001$), and total extents of mineralisation ($F = 26.117$, $p < 0.001$) were

279 significantly greater in all root-amended treatments compared to the control, with the highest
280 values measured in *L. perenne* condition.

281 The effect of possible interactions between PAHs, diesel and activated charcoal was also
282 measured (Figure 5). At the first time point (Figure 5a), *M. sativa* and *S. bicolor* amended
283 treatments showed significantly shorter lag phases (F = 19.154, p = 0.003). Moreover, total
284 extents of mineralisation were significantly reduced by the presence of roots of any species
285 (F = 21.273, p = 0.002), while maximum rates of mineralisation were not affected by the
286 addition of roots. After 14 d (Figure 5b), the lag phases were significantly different (F =
287 14.017, p < 0.001), being shorter in both the soil amended with *L. perenne*, as well as the
288 non-amended soil. Maximum rates were also influenced by the treatments; exhibiting highest
289 maximum rates when roots of any of the species were added to the soil (F =11.381, p =
290 0.001). In contrast, total extents of mineralisation were unaffected by the different treatments.
291 After 28 d incubations, all mineralisation measurements: lag phases (F = 17.077, p < 0.001),
292 maximum rates (F = 47.729, p < 0.001), and total extents (F = 11.832, p = 0.001) were
293 significantly enhanced by the addition of any of the roots (Figure 5c).

294 Controls measuring the mineralisation of ^{14}C-phenanthrene in the absence of ^{12}C-PAHs were
295 also assessed in pristine soil, as well as in soils containing activated charcoal and diesel
296 amendments (SI 4). Overall, controls behaved in a similar way after 1 and 14 d incubation.
297 Most significant differences were observed after 14 d, where soil without root amendment
298 often displayed shorter lag phases, especially when *L. perenne* was added. Such are the cases
299 of the pristine soil (F = 13.645, p = 0.002), soil with activated charcoal (F = 246.58, p <
300 0.001), and solvent amended soil (F = 22.061, p < 0.001). However, after 28d incubation,
301 maximum rates of mineralisation were significantly faster when *L. perenne* was used as
302 amendment in pristine (F = 4.592, p = 0.033), diesel (F = 3.935, p = 0.023), and the mixture
303 of activated charcoal and diesel controls (F = 4.618, p = 0.035). Total extents of

304 mineralisation were also significantly higher in three of the controls by the addition of roots
305 from *L. perenne*, with the exception of the control amended with a mixture of diesel and
306 activated charcoal ($F = 1.106$, $p = 0.417$). Lag phases presented differences in all treatments,
307 with significantly shorter values in the absence of roots for the pristine soil ($F = 160.66$, $p <$
308 0.001), and individual amendments of diesel ($F = 11.683$, $p = 0.001$) and activated charcoal
309 ($F = 227.18$, $p < 0.001$).

310 3.3 Enumeration of bacteria

311 Microbial numbers were significantly influenced by all treatments and conditions ($p < 0.05$).
312 For both total heterotrophic and phenanthrene degrading bacteria (CFU g^{-1}soil dw)
313 significantly increased after the addition of ^{14}C-phenanthrene at all time points ($p < 0.01$),
314 with the exception of the phenanthrene degrading bacteria at the last sampling time (28 d).

315 The numbers of total heterotrophic bacteria in soil before the mineralisation assay were
316 influenced by the different contaminant conditions, as well as by the root amendments at the
317 different time points (SI 5). One day after spiking, the control pristine soil showed that root
318 amendments increased the numbers of total heterotrophic bacteria ($F = 131.952$, $p < 0.001$),
319 especially by *G. max*. This same effect was also observed in treatments containing diesel
320 (Treatments C and D). After 14 d, total heterotrophic bacteria numbers were significantly
321 reduced compared to the first time point ($t = 5.774$, $p < 0.001$). CFUs from pristine soil
322 displayed the largest reduction, especially when roots from *L. perenne* and *G. max* were
323 present. Furthermore, all soil conditions displayed the highest microbial numbers in the
324 presence of either one of the grass species (*L. perenne* or *S. bicolor*). Finally, after 28 d
325 incubation, total heterotrophic bacterial numbers were not significantly different to those
326 measured after 14 d ($t = -1.846$, $p = 0.068$).

Numbers of phenanthrene degrading bacteria were also significantly influenced by the different soil conditions and amendment with root biomass ($p < 0.05$). One day after spiking, roots from legumes (*M. sativa* and *G. max*) increased the CFUs in the grass- and non-amended treatments with the exception of the diesel and solvent controls. After 14 d incubation, most soil conditions excluding the mixture of PAHs, activated charcoal and diesel (Treatment D), led to significantly higher numbers of phenanthrene degrading bacteria in the presence of roots ($p < 0.05$). After 28 d incubation, the same trend was observed with decreased microbial numbers in the plant-free controls and an enhanced presence of bacteria in soil amended with *M. sativa* root biomass ($p < 0.05$).

Overall CFU total heterotrophic bacteria from "respirometers" significantly decreased in number over time ($p < 0.05$). Initially, all treatments contained significantly higher CFU numbers ($p < 0.05$) in the presence of plant roots, exhibiting consistently higher values when *M. sativa* was used. After 14 d, the opposite trend was observed, with the exception of the treatments containing activated charcoal (Treatments B and D), where root-amended soil presented increased microbial numbers but without specific pattern regarding plant identity. This effect was also observed only in the control amended with diesel (Treatment G). After the 28d sampling, root amendments showed to significantly enhance three out of the nine conditions. This trend was observed on the treatment containing PAHs and activated charcoal (Treatment B, $F = 376.55$, $p = 0.001$), the one including a mixture of PAHs, diesel and activated charcoal (Treatment D, $F = 22.089$, $p = 0.002$), and the control for diesel (Treatment G, $F = 17.493$, $p = 0.004$). In the rest of the conditions, as observed before, root amendments reduced the total heterotrophic CFUs.

The phenanthrene degrading bacteria from "respirometers" was only affected by the plant amendments in the first two sampling points (1 and 14d). In both cases, *L. perenne* roots consistently produced a significantly larger number of CFUs ($p < 0.05$). At the final sampling

point of the mineralisation assay (28d), treatments without root amendments presented significantly less CFU than any of the root amended treatments ($p < 0.05$). Overall results also showed that *L. perenne* and *M. sativa* amendments produced the highest CFUs numbers across the different soil conditions.

4. Discussion

4.1 Phytotoxicity of PAH-NAPL contaminated soil

Different phytotoxicity parameters can be used to assess the ability of a plant to germinate and grow in PAH contaminated soil (Maila and Cloete, 2002). For instance, seed emergence cannot always be used as a reliable endpoint of phytotoxicity, as seeds typically obtain nutrients from internal materials at the earliest stages of germination. Nevertheless, when diesel is included, the low molecular weight fractions have been shown to have inhibitory effects on the germination of some species (Henner et al., 1999). In the current study, these effects were only observed for *T. repens*. This overall lack of toxicity has also been observed previously by Smith et al. (2006), who found that germination of different species (including *T. repens*) was unaffected by the presence of these fractions. The absence of phytotoxic effects of hydrocarbons (especially PAHs) on germination / seed emergence of different species has been also reported by multiple authors, including Hamdi et al. (2012) and Smreckzak and Maliszewska-Kordybach (2003), who also attributed this lack of toxicity to the low bioavailability of these hydrophobic chemicals.

The phytotoxicity assessment, based on seeds survival, showed that *S. bicolor* was the most suitable species to be grown in a PAH-NAPL amended soil. Previous studies have shown similar results; for example, Muratova et al. (2009) observed an increased survival of *S. bicolor* at 10 mg kg^{-1} phenanthrene compared to the control uncontaminated soil. This result gains importance considering that *S. bicolor* has also been found to enhance the bioremediation process of crude oil contaminated soil (Banks et al., 2003). As a consequence, this species would be able to both germinate and grow in contaminated soil while promoting the remediation of contaminated soil.

Although PAH contaminated soil is generally assumed as toxic for most plant species, there are some reported cases where PAHs have been found to have enhancing effects on plant

381 growth. For instance, Maliszewska-Kordybach and Smreczak (2000) observed this trend,
382 reflected on increased root growth of certain grass species. The authors described this
383 behaviour as a feedback control mechanism, which in this case is reflected as an enhanced
384 growth of plants as a response to be exposed to PAHs (Maliszewska-Kordybach and
385 Smreczak, 2000). Overall, the lack of consensus regarding the effect of PAHs on germination
386 and growth of plant species could be attributed to the natural variability on seed viability, as
387 well as the specific characteristics of the soil used for the test. These factors have already
388 been suggested by different authors such as Maliszewska-Kordybach and Smreczak (2000),
389 who concluded that soils with sandy textures had greater phytotoxic effects on most of the
390 tested plant species. Additionally other soil properties including organic carbon content,
391 cation-exchange capacity, or specific surface area will also play an important role on the fate
392 and behaviour of organic pollutants (Chung and Alexander, 2002). In the present study,
393 overall seed survival remained unaffected by the presence of PAHs in *L. perenne*, *S. bicolor*,
394 *M. sativa* and *G.max*. The lack of phytotoxic effects of the mixture of PAHs might be the
395 result of the combination of two main factors. First, these four species presented a higher
396 PAH and NAPL tolerance, which has also been reported before (Banks et al., 2003; Gao and
397 Zhu, 2004; Muratova et al., 2003; Phillips et al., 2006). And second, the soil used has been
398 defined as high-content silt and clay soil, which might be producing a higher rate and extent
399 of the pollutant's sequestration hence reducing its bioavailability.

400 4.2 Effect of root amendments in the mineralisation of ^{14}C-phenanthrene
401 When root biomass from different plant species was added to PAHs contaminated soil, the
402 impact on the mineralisation of ^{14}C-phenanthrene became significantly higher after 2 weeks
403 incubation for soil contaminated only with PAHs. When diesel was also present in the
404 mixture (PAH-NAPL), this behaviour was only observed after 4 reaching in some cases total
405 extents of mineralisation close to 100%. In all cases, studies reporting this same trend have

406 been consistently reported, especially using *L. perenne* and *M. sativa*. These two species have
407 been found to be effective enhancers of the dissipation of PAHs from soil by several authors
408 such as Binet et al. (2000), Joner et al. (2001) or Phillips et al. (2009). Other authors have
409 also observed that a higher biodegradation of hydrocarbons can be achieved by the use of
410 plants, either as a whole or by the use of part of them (*e.g.* root biomass)(Miya and Firestone,
411 2001; Rentz et al., 2005; Smith et al., 2011; Sun et al., 2011). Moreover, when ^{14}C isotopes
412 were used, similar results have also been observed. For instance, Reilley et al. (1996)
413 consistently found higher mineralisation of ^{14}C-pyrene in simulated rhizospheric soil.
414 Additionally, the temporal factor observed in this study has also been observed by other
415 authors, reporting an initial inhibition on the mineralisation of ^{14}C-pyrene, followed by an
416 increased biodegradation after 100 d of incubation (Mueller and Shann, 2007). This same
417 behaviour was also observed by Macleod and Semple (2002), who observed that microbes
418 required an adaptation period in order to biodegrade ^{14}C-pyrene. In this case, authors
419 suggested that this behaviour could indicate that period of microbial growth was necessary
420 before mineralisation started (Macleod and Semple, 2002).
421 Even though the specific processes producing this enhanced dissipation of hydrocarbons from
422 soil are not completely understood, the overall consensus is that this effect is due to increases
423 in the numbers of pollutant degrading microbes when plants are introduced into contaminated
424 soil – termed the "rhizosphere effect" (Aprill and Sims, 1990; Binet et al., 2000; Chiapusio et
425 al., 2007; Fan et al., 2008; Günther et al., 1996; Radwan et al., 1998). However, measurement
426 of bacterial numbers in this study did not show a significant positive relationship with ^{14}C-
427 phenanthrene mineralisation for either total heterotrophic or phenanthrene-degrading
428 bacteria. Nonetheless, a similar decreasing trend has been reported previously by Nichols et
429 al. (1997), concluding that this effect could be the result of an environment with limited
430 amounts of accessible carbon and other nutrients. Another reason for this trend could be
431 related to a priming effect, which can be characterized by a fast increase of the microbial

populations after a change in the system (soil spiking and amendment) followed by a reduction of the microbial numbers after every time point (Kuzyakov, 2002). *M. sativa* was the only species that produced higher CFUs of phenanthrene degrading bacteria after 14 and 28 d incubation, but with no visible pattern among treatments. This specific behaviour produced by *M. sativa* has previously been reported by Phillips et al. (2006), who observed a positive correlation between the mineralisation of ^{14}C-phenanthrene and microbial numbers from soil planted with *M. sativa*. Given the overall findings, the observed higher ^{14}C-mineralisation from soil amended with roots could be related to a putative increase of the bioavailability of the contaminant, but this was not measured directly in this study. However, a similar conclusion has also been proposed by Joner et al. (2002), who suggested that rhizospheric soil could produce a change in surface adsorption processes controlling the fate of hydrocarbons in soil (Joner et al., 2002; Reilley et al., 1996). Furthermore, authors hypothesized that substances produced by roots due to exudation, decay and turnover might also have a contributing factor in the biodegradation process (Mueller and Shann, 2007; Rentz et al., 2005).

5. Conclusions

The phytotoxicity findings reported in this study show that the grasses (*L. perenne* and *S. bicolor*) and legumes (*M. sativa* and *G. max*) are suitable for plant-enhanced biodegradation, as these are capable of germinating and growing in contaminated soil without showing detrimental effects. When these four species were tested to assess the effect of root tissue on the mineralisation of ^{14}C-phenanthrene, all species produced enhanced mineralisation after two and four weeks since root biomass amendment. This suggests that root amended soil has greater potential to mineralise ^{14}C-phenanthrene than soils lacking plants or root biomass. Microbial numbers of both heterotrophs and phenanthrene degrading bacteria did not appear to have a significant relationship with the mineralisation parameters. The total CFUs were reduced over time despite the increasing extents in the mineralisation of ^{14}C-phenanthrene. Results from the present study suggest that the increased mineralisation of ^{14}C-phenanthrene might be the result of (1) the enhancement of the bioavailability of the contaminant as well as (2) the contribution of readily available C and nutrients from root exudation, decay and turnover, rather than to an increase on the number of bacteria capable to degrade PAHs as is generally assumed.

Acknowledgements

GVC would like to thank the Mexican National Council for Science and Technology (CONACyT) for their financial support through a scholarship (No. 313807) to pursue postgraduate studies at the Lancaster Environment Centre, Lancaster University.

467 References

468 Anderson, T.A., Guthrie, E.A., Walton, B.T., 1993. Bioremediation in the rhizosphere.
469 Environ. Sci. Technol. 27, 2630-2636.

470 Aprill, W., Sims, R.C., 1990. Evaluation of the use of prairie grasses for stimulating
471 polycyclic aromatic hydrocarbon treatment in soil. Chemosphere 20, 253-265.

472 Baek, K.-H., Kim, H.-S., Oh, H.-M., Yoon, B.-D., Kim, J., Lee, I.-S., 2004. Effects of crude
473 oil, oil components, and bioremediation on plant growth. J. Environ. Sci. Health A Tox.
474 Hazard Subst. Environ. Eng. 39, 2465-2472.

475 Banks, M.K., Kulakow, P., Schwab, A.P., Chen, Z., Rathbone, K., 2003. Degradation of
476 crude oil in the rhizosphere of Sorghum bicolor. Int. J. Phytoremediat. 5, 225-234.

477 Binet, P., Portal, J.M., Leyval, C., 2000. Dissipation of 3-6-ring polycyclic aromatic
478 hydrocarbons in the rhizosphere of ryegrass. Soil Biol. Biochem. 32, 2011-2017.

479 Chiapusio, G., Pujol, S., Toussaint, M.L., Badot, P.M., Binet, P., 2007. Phenanthrene toxicity
480 and dissipation in rhizosphere of grassland plants (Lolium perenne L. and Trifolium pratense
481 L.) in three spiked soils. Plant Soil 294, 103-112.

482 Chung, N., Alexander, M., 2002. Effect of soil properties on bioavailability and extractability
483 of phenanthrene and atrazine sequestered in soil. Chemosphere 48, 109-115.

484 Corgié, S.C., Joner, E.J., Leyval, C., 2003. Rhizospheric degradation of phenanthrene is a
485 function of proximity to roots. Plant Soil 257, 143 - 150.

486 Doick, K.J., Lee, P.H., Semple, K.T., 2003. Assessment of spiking procedures for the
487 introduction of a phenanthrene- LNAPL mixture into field- wet soil. Environ. Pollut. 126,
488 399-406.

489 Doick, K.J., Semple, K.T., 2003. The effect of soil: water ratios on the mineralisation of
490 phenanthrene: LNAPL mixtures in soil. FEMS Microbiol. Lett. 220, 29-33.

491 Fan, S., Li, P., Gong, Z., Ren, W., He, N., 2008. Promotion of pyrene degradation in
492 rhizosphere of alfalfa (Medicago sativa L.). Chemosphere 71, 1593-1598.

493 Fenlon, K.A., Jones, K.C., Semple, K.T., 2007. Development of microbial degradation of
494 cypermethrin and diazinon in organically and conventionally managed soils. J. Environ.
495 Monit. 9, 510-515.

496 Gao, Y., Zhu, L., 2004. Plant uptake, accumulation and translocation of phenanthrene and
497 pyrene in soils. Chemosphere 55, 1169-1178.

498 Günther, T., Dornberger, U., Fritsche, W., 1996. Effects of ryegrass on biodegradation of
499 hydrocarbons in soil. Chemosphere 33, 203-215.

500 Hamdi, H., Benzarti, S., Aoyama, I., Jedidi, N., 2012. Rehabilitation of degraded soils
501 containing aged PAHs based on phytoremediation with alfalfa (Medicago sativa L.). Int.
502 Biodeterior. Biodegradation 67, 40-47.

503 Henner, P., Schiavon, M., Druelle, V., Lichtfouse, E., 1999. Phytotoxicity of ancient gaswork
504 soils. Effect of polycyclic aromatic hydrocarbons (PAHs) on plant germination. Org.
505 Geochem. 30, 963-969.

506 Joner, E.J., Corgié, S.C., Amellal, N., Leyval, C., 2002. Nutritional constraints to degradation
507 of polycyclic aromatic hydrocarbons in a simulated rhizosphere. Soil Biol. Biochem. 34, 859-
508 864.

509 Joner, E.J., Johansen, A., Loibner, A.P., dela Cruz, M.A., Szolar, O.H.J., Portal, J.M., Leyval,
510 C., 2001. Rhizosphere effects on microbial community structure and dissipation and toxicity
511 of polycyclic aromatic hydrocarbons (PAHs) in spiked soil. Environ. Sci. Technol. 35, 2773-
512 2777.

513 Kuzyakov, Y., 2002. Review: Factors affecting rhizosphere priming effects. J. Plant Nutr.
514 Soil Sci. 165, 382-396.

515 Langer, I., Syafruddin, S., Steinkellner, S., Puschenreiter, M., Wenzel, W., 2010. Plant
516 growth and root morphology of Phaseolus vulgaris L. grown in a split-root system is affected
517 by heterogeneity of crude oil pollution and mycorrhizal colonization. Plant Soil 332, 339-
518 355.

519 Macleod, C.J.A., Semple, K.T., 2002. The adaptation of two similar soils to pyrene
520 catabolism. Environ. Pollut. 119, 357-364.

521 Maila, M.P., Cloete, T.E., 2002. Germination of Lepidium sativum as a method to evaluate
522 polycyclic aromatic hydrocarbons (PAHs) removal from contaminated soil. Int. Biodeterior.
523 Biodegradation 50, 107-113.

524 Maliszewska-Kordybach, B., Smreczak, B., 2000. Ecotoxicological activity of soils polluted
525 with polycyclic aromatic hydrocarbons (PAHs) - Effect on plants. Environ. Technol. 21,
526 1099-1110.

527 Miya, R.K., Firestone, M.K., 2001. Enhanced phenanthrene biodegradation in soil by slender
528 oat root exudates and root debris. J. Environ. Qual. 30, 1911-1918.

529 Moubasher, H.A., Hegazy, A.K., Mohamed, N.H., Moustafa, Y.M., Kabiel, H.F., Hamad,
530 A.A., 2015. Phytoremediation of soils polluted with crude petroleum oil using Bassia
531 scoparia and its associated rhizosphere microorganisms. Int. Biodeterior. Biodegradation 98,
532 113-120.

533 Mueller, K.E., Shann, J.R., 2007. Effects of tree root-derived substrates and inorganic
534 nutrients on pyrene mineralization in rhizosphere and bulk soil. J. Environ. Qual. 36, 120-
535 127.

536 Muratova, A., Dmitrieva, T., Panchenko, L., Turkovskaya, O., 2008. Phytoremediation of oil-
537 sludge–contaminated soil. Int. J. Phytoremediat. 10, 486-502.

538 Muratova, A., Golubev, S., Wittenmayer, L., Dmitrieva, T., Bondarenkova, A., Hirche, F.,
539 Merbach, W., Turkovskaya, O., 2009. Effect of the polycyclic aromatic hydrocarbon
540 phenanthrene on root exudation of Sorghum bicolor (L.) Moench. Environ. Exp. Bot. 66,
541 514-521.

542 Muratova, A., Hübner, T., Tischer, S., Turkovskaya, O., Möder, M., Kuschk, P., 2003. Plant–
543 rhizosphere-microflora association during phytoremediation of PAH-contaminated soil. Int. J.
544 Phytoremediat. 5, 137-151.

545 Nichols, T.D., Wolf, D.C., Rogers, H.B., Beyrouty, C.A., Reynolds, C.M., 1997. Rhizosphere
546 microbial populations in contaminated soils. Water Air Soil Pollut. 95, 165-178.

547 OECD, 2003. Terrestrial Plant test. 208: Seedling Emergence and Seedling Growth Test, In:
548 Development, O.f.E.C.-o.a. (Ed.). Organisation for Economic Co-operation and
549 Development, p. 19.

550 Olson, P.E., Castro, A., Joern, M., DuTeau, N.M., Pilon-Smits, E.A.H., Reardon, K.F., 2007.
551 Comparison of plant families in a greenhouse phytoremediation study on an aged polycyclic
552 aromatic hydrocarbon-contaminated soil. J. Environ. Qual. 36, 1461-1469.

553 Oyelami, A.O., Okere, U.V., Orwin, K.H., De Deyn, G.B., Jones, K.C., Semple, K.T., 2013.
554 Effects of plant species identity, diversity and soil fertility on biodegradation of phenanthrene
555 in soil. Environ. Pollut. 173, 231-237.

556 Phillips, L.A., Greer, C.W., Farrell, R.E., Germida, J.J., 2009. Field-scale assessment of
557 weathered hydrocarbon degradation by mixed and single plant treatments. Appl. Soil Ecol.
558 42, 9-17.

559 Phillips, L.A., Greer, C.W., Germida, J.J., 2006. Culture- based and culture- independent
560 assessment of the impact of mixed and single plant treatments on rhizosphere microbial
561 communities in hydrocarbon contaminated flare- pit soil. Soil Biol. Biochem. 38, 2823-2833.

562 Pilon-Smits, E., 2005. Phytoremediation. Annu. Rev. Plant Biol. 56, 15-39.

563 Radwan, S.S., Al-Awadhi, H., Sokhoh, H.A., El-Nemr, I.M., 1998. Rhizospheric
564 hydrocarbon-utilizing microorganisms as potential contributors to phytoremediation for the
565 oily Kuwaiti desert. Microbiol. Res. 153, 247-251.

566 Reilley, K.A., Banks, M.K., Schwab, A.P., 1996. Dissipation of polycyclic aromatic
567 hydrocarbons in the rhizosphere. J. Environ. Qual. 25, 212-219.

568 Rentz, J.A., Alvarez, P.J.J., Schnoor, J.L., 2005. Benzo[a] pyrene co-metabolism in the
569 presence of plant root extracts and exudates: Implications for phytoremediation. Environ.
570 Pollut. 136, 477-484.

571 Rezek, J., in der Wiesche, C., Mackova, M., Zadrazil, F., Macek, T., 2008. The effect of
572 ryegrass (Lolium perenne) on decrease of PAH content in long term contaminated soil.
573 Chemosphere 70, 1603-1608.

574 Rhodes, A.H., Carlin, A., Semple, K.T., 2008. Impact of black carbon in the extraction and
575 mineralization of phenanthrene in soil. Environ. Sci. Technol. 42, 740-745.

576 Rorison, H., Robinson, D., 1986. Mineral Nutrition, In: Moore, P.D., Chapman, S.B. (Eds.),
577 Methods in Plant Ecology. Blackwell Scientific Publications, United Kingdom, pp. 145 - 214.

578 Smith, M.J., Flowers, T.H., Duncan, H.J., Alder, J., 2006. Effects of polycyclic aromatic
579 hydrocarbons on germination and subsequent growth of grasses and legumes in freshly
580 contaminated soil and soil with aged PAHs residues. Environ. Pollut. 141, 519-525.

581 Smith, M.J., Flowers, T.H., Duncan, H.J., Saito, H., 2011. Study of PAH dissipation and
582 phytoremediation in soils: comparing freshly spiked with weathered soil from a former
583 coking works. J. Hazard. Mater. 192, 1219-1225.

584 Smreckzak, B., Maliszewska-Kordybach, B., 2003. Seeds germination and root growth of
585 selected plants in pah contaminated soil. Fresen. Environ. Bull. 12, 946-949.

586 Sun, M., Fu, D., Teng, Y., Shen, Y., Luo, Y., Li, Z., Christie, P., 2011. In situ
587 phytoremediation of PAH-contaminated soil by intercropping alfalfa (Medicago sativa L.)
588 with tall fescue (Festuca arundinacea Schreb.) and associated soil microbial activity. J. Soils
589 Sediments 11, 980-989.

590 Thompson, O.A., Wolf, D.C., Mattice, J.D., Thoma, G.J., 2008. Influence of nitrogen
591 addition and plant root parameters on phytoremediation of pyrene- contaminated soil. Water
592 Air Soil Pollut. 189, 37-47.

593 Towell, M.G., Browne, L.A., Paton, G.I., Semple, K.T., 2011. Impact of carbon
594 nanomaterials on the behaviour of C-14-phenanthrene and C-14-benzo-[a] pyrene in soil.
595 Environ. Pollut. 159, 706-715.

596 Trapp, S., Karlson, U., 2001. Aspects of phytoremediation of organic pollutants. J Soils &
597 Sediments 1, 37-43.

598 US EPA, U.S.E.P.A., 2012. 850.4100 - Seedling Emergence and Seedling Growth, In:
599 Agency, U.S.E.P. (Ed.), United States, p. 32.

600 Wenzel, W., 2009. Rhizosphere processes and management in plant-assisted bioremediation
601 (phytoremediation) of soils. Plant Soil 321, 385-408.

602 Yoshitomi, K.J., Shann, J.R., 2001. Corn (Zea mays L.) root exudates and their impact on
603 14C-pyrene mineralization. Soil Biol. Biochem. 33, 1769-1776.
604

605 Tables

606

607 Table 1. Test plant species

Family	Species
Pocacea	*Lolium perenne**
	Festuca rubra
	*Sorghum bicolor**
Fabaceae	*Medicago sativa**
	Trifolium repens
	*Glycine max**
Asteraceae	*Achillea millefolium*
Onagraceae	*Chamerion angustifolium*
Plantaginaceae	*Plantago lanceolata*

*Species used for both phytotoxicity and mineralisation assay

608

609 Table 2. Summary of treatments for the phytotoxicity assay

Treatment	Characteristics
T1	100 mg kg^{-1} Σ PAH + 0.1% (w/w) diesel
T2	100 mg kg^{-1} Σ PAH + 0.1 % (dry weight) activated charcoal
T3	300 mg kg^{-1} Σ PAH + 0.1% (w/w) diesel
T4	300 mg kg^{-1} Σ PAH + 0.1 % (dry weight) activated charcoal
T5	100 mg kg^{-1} Σ PAH + 0.1% (w/w) diesel + 0.1 % (dry weight) activated charcoal
T6	300 mg kg^{-1} Σ PAH + 0.1% (w/w) diesel + 0.1 % (dry weight) activated charcoal
T7	100 mg kg^{-1} Σ PAH
T8	300 mg kg^{-1} Σ PAH
C_d	Diesel control: 0.1% (w/w) diesel
C_{ac}	Activated charcoal control: 0.1 % (dry weight) activated charcoal
C_s	Solvent control: 10 ml kg^{-1} 1:1 toluene:acetone
C_g	Growth control: Clean soil

610

611 Table 3. Summary of treatments for the ^{14}C-phenanthrene mineralisation assay

Treatment	Characteristics
A	100 mg kg^{-1} Σ PAH
B	100 mg kg^{-1} Σ PAH + 0.1 % (dry weight) activated charcoal
C	100 mg kg^{-1} Σ PAH + 0.1% (w/w) diesel
D	100 mg kg^{-1} Σ PAH + 0.1% (w/w) diesel + 0.1 % (dry weight) activated charcoal
E	Pristine soil
F	0.1 % (dry weight) activated charcoal
G	0.1% (w/w) diesel
H	0.1% (w/w) diesel + 0.1 % (dry weight) activated charcoal
I	1:1 Toluene : Acetone solution

612

613

Figure captions

Figure 1 Germination rate estimated at the 7th day after sowing as the percentage of germinated seeds among the seeds sown (Muratova et al. 2008). T1 = 100 mg kg^{-1} Σ PAH + 0.1% (w/w) diesel, T2 = 100 mg kg^{-1} Σ PAH + 0.1 % (dry weight) activated charcoal, T3 = 300 mg kg^{-1} Σ PAH + 0.1% (w/w) diesel, T4 = 300 mg kg^{-1} Σ PAH + 0.1 % (dry weight) activated charcoal, T5 = 100 mg kg^{-1} Σ PAH + 0.1% (w/w) diesel + 0.1 % (dry weight) activated charcoal, T6 = 300 mg kg^{-1} Σ PAH + 0.1% (w/w) diesel + 0.1 % (dry weight) activated charcoal, T7 = 100 mg kg^{-1} Σ PAH, T8 = 300 mg kg^{-1} Σ PAH, C_d = Diesel control: 0.1% (w/w) diesel, C_{ac} = Activated charcoal control: 0.1 % (dry weight) activated charcoal, C_s = Solvent control: 10 ml kg^{-1} 1:1 toluene:acetone, C_g = Growth control: Clean soil. Marked boxes (*) represent significant differences against the growth control (C_g) of each species. Solid lines represent the mean value of the growth control (Cg) used as baseline for the analysis of variance (ANOVA) test. Error bars represent the standard error of the mean (*n* = 3)

Figure 2 Mineralisation of ^{14}C-phenanthrene in soil spiked with 100 mg kg^{-1} Σ PAH, Root amendments: (•) *L. perenne*, (○) S. *bicolor*, (▼) *M. sativa*, (▽) *G. max*, and (■) without roots after 1 (**a**), 14 (**b**), and 28 (**c**) days ageing. Error bars represent the standard error of the mean (*n* = 3).

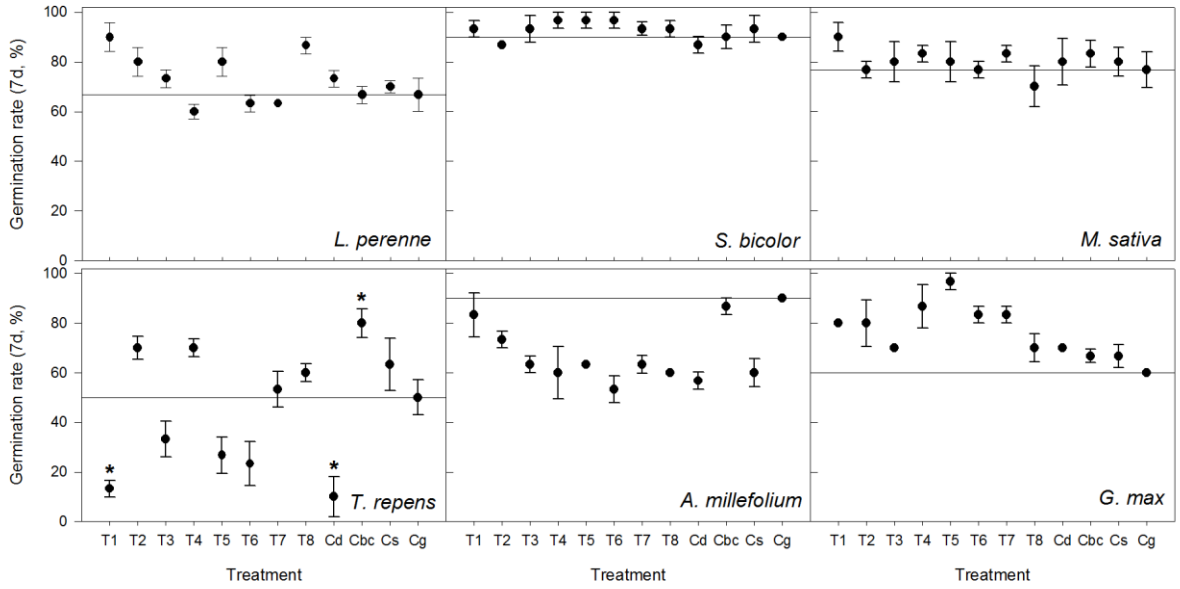
Figure 3 Mineralisation of ^{14}C-phenanthrene in soil spiked with 100 mg kg^{-1} Σ PAH and 0.1% activated charcoal. Root amendments: (•) *L. perenne*, (○) *S. bicolor*, (▼) *M. sativa*, (▽) *G. max*, and (■) without roots after 1 (**a**), 14 (**b**), and 28 (**c**) days ageing. Error bars represent the standard error of the mean (*n* = 3)

Figure 4 Mineralisation of ^{14}C-phenanthrene in soil spiked with 100 mg kg^{-1} Σ PAH and 0.1% diesel. Root amendments: (•) *L. perenne*, (○) *S. bicolor*, (▼) *M. sativa*, (▽) *G. max*, and (■) without roots after 1 (**a**), 14 (**b**), and 28 (**c**) days ageing. Error bars represent the standard error of the mean (*n* = 3)

Figure 5 Mineralisation of ^{14}C-phenanthrene in soil spiked with 100 mg kg^{-1} Σ PAH, 0.1% activated charcoal (dw) and 0.1% diesel (w/w). Root amendments: (•) *L. perenne*, (○) *S. bicolor*, (▼) *M. sativa*, (▽) *G. max*, and (■) without roots after 1 (**a**), 14 (**b**), and 28 (**c**) days ageing. Error bars represent the standard error of the mean (*n* = 3)

644

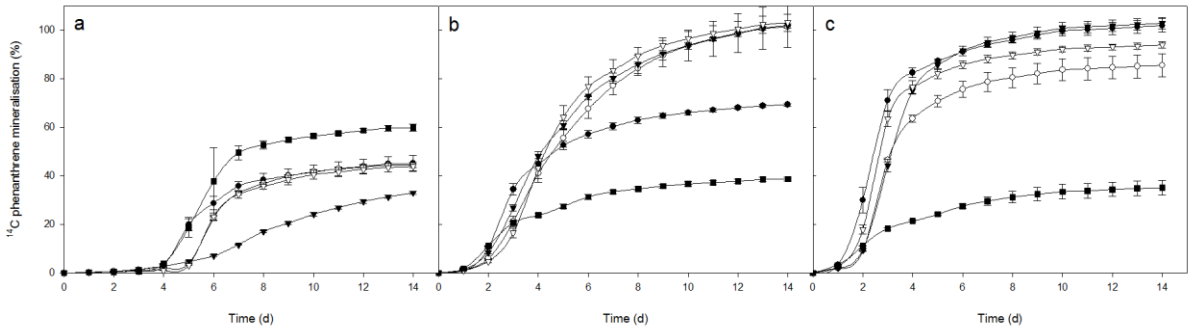
645 Figure 1



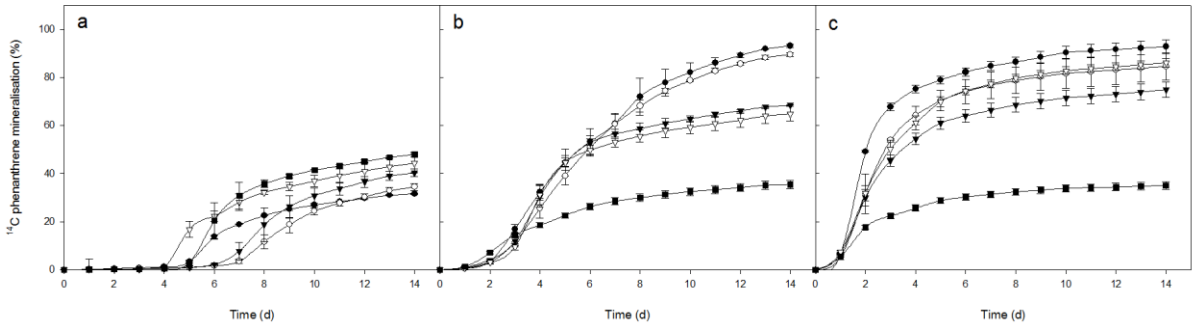
646

647

Figure 2



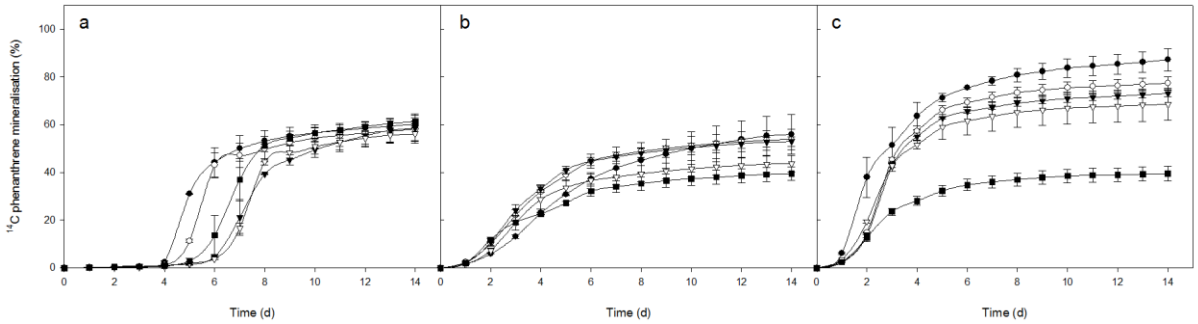
651 Figure 3



652

653

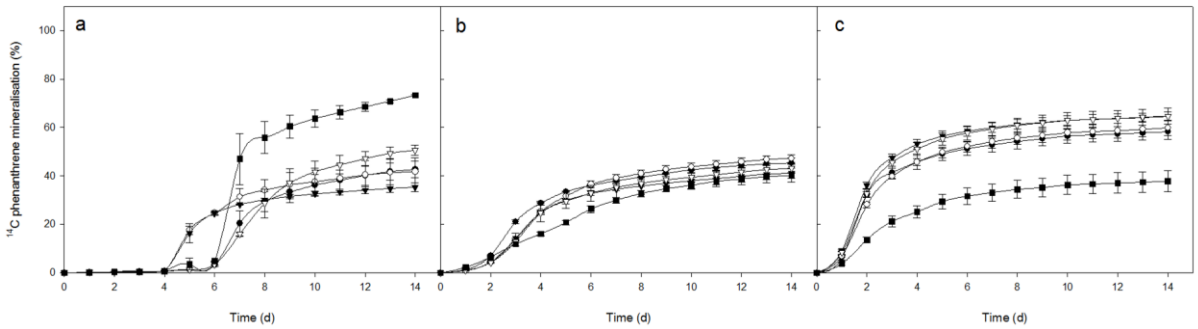
654 Figure 4



655

656

657 Figure 5



658