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REGULAR ARTICLE

Indirect effects of polycyclic aromatic hydrocarbon contamination on microbial communities in legume and grass rhizospheres

Akitomo Kawasaki · Erika R. Watson · Michael A. Kertesz

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Abstract
Background and aims Biodegradation of polycyclic aromatic hydrocarbons (PAHs) is accelerated in the presence of plants, due to the stimulation of rhizosphere microbes by plant exudates (nonspecific enhancement). However, plants may also recruit specific microbial groups in response to PAH stress (specific enhancement). In this study, plant effects on the development of rhizosphere microbial communities in heterogeneously contaminated soils were assessed for three grasses (ryegrass, red fescue and Yorkshire fog) and four legumes (white clover, chickpea, subterranean clover and red lentil).
Methods Plants were cultivated using a split-root model with their roots divided between two independent pots containing either uncontaminated soil or PAH-contaminated soil (pyrene or phenanthrene). Microbial community development in the two halves of the rhizosphere was assessed by T-RFLP (bacterial and fungal community) or DGGE (bacterial community), and by 16S rRNA gene tag-pyrosequencing.
Results In legume rhizospheres, the microbial community structure in the uncontaminated part of the split-root model was significantly influenced by the presence of PAH-contamination in the other part of the root system (indirect effect), but this effect was not seen for grasses. In the contaminated rhizospheres, *Verrucomicrobia* and *Actinobacteria* showed increased populations, and there was a dramatic increase in *Denitratisoma* numbers, suggesting that this genus may be important in rhizoremediation processes.
Conclusion Our results show that *Trifolium* and other legumes respond to PAH-contamination stress in a systemic manner, to influence the microbial diversity in their rhizospheres.

Keywords Polycyclic aromatic hydrocarbon · Rhizoremediation · Rhizosphere · Legume · DGGE · Microbial community

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Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of hazardous organic contaminants which are largely anthropogenic in nature and are ubiquitous in the environment, especially in industrial soils (Habe and Omori 2003; Johnsen et al. 2005). Some of these compounds are known human carcinogens (Schneider et al. 2002) and 16 PAHs have been prioritized by the United States Environmental Protection Agency (USEPA) for environmental regulation (Habe and Omori 2003). PAH-contaminated soils can be remediated by a variety

of physical and chemical processes, but microbial biodegradation is increasingly the remediation method of choice (Haritash and Kaushik 2009).

Enhanced degradation of organic contaminants including PAHs is often observed in plant rhizospheres, a remediation process known as rhizoremediation (Anderson et al. 1993; Kuiper et al. 2004). Compared to more traditional bioremediation techniques such as biostimulation and bioaugmentation, rhizoremediation possesses several advantages. Rhizoremediation processes are driven by the native microbial community colonizing the rhizosphere (Kuiper et al. 2004), which is generally two to four orders of magnitude greater than in the surrounding bulk soil (Salt et al. 1998). These populations are supported by the plants, which release 10–20% of their photosynthate as root exudates containing sugars, amino acids and proteins that serve as nutrient sources for the rhizosphere microbial community (Kuiper et al. 2004; Salt et al. 1998). Surfactant-active compounds in the root exudates increase the solubility of the contaminant and make it more bioavailable to root colonizing microorganisms. In addition, the soil water flux towards the roots, driven by plant transpiration, provides an effective means by which the contaminant is translocated to the microbial community in the rhizosphere (Child et al. 2007), and higher PAH degradation is observed closer to the root surface (Corgie et al. 2003).

Various studies have demonstrated the effectiveness of rhizoremediation in PAH-contaminated soils, but the efficiency of the process varies greatly with plant species (Lee et al. 2008; Siciliano et al. 2003). Grass species are generally suitable for rhizoremediation because they possess a large root biomass that harbours a higher total microbial population than other species of a comparable size (Chiapusio et al. 2007). In ryegrass rhizosphere, removal of eight PAHs ranging from three to six aromatic rings in size was significantly higher than in unvegetated soil, and the number of culturable PAH degraders was also increased (Binet et al. 2000). Leguminous plants are also well studied for rhizoremediation of PAH-contaminated soils because of their ability to fix atmospheric nitrogen. In alfalfa rhizosphere, for example, removal of anthracene was enhanced 28% compared to the unplanted control soil, and alfalfa root exudates were shown to increase the solubilization of anthracene by 50% (Kim et al. 2004), confirming the important part that the plant plays in increasing pollutant bioavailability to the soil microbes.

Root colonizing microorganisms play a major role in the rhizoremediation process, but the complex interactions that exist between plants and microbes are not fully understood (Siciliano and Germida 1998). The simplest explanation of these interactions is that PAH degradation in the rhizosphere is enhanced "nonspecifically" by the rhizosphere effect, i.e. higher microbial activity in the rhizosphere compared to the bulk soil affords higher PAH-degradation activity (Siciliano et al. 2003). Plant root exudates contain many substrates which are chemically similar to pollutants, and these substrates may act as pollutant analogues to nonspecifically enhance the rhizoremediation process (Siciliano and Germida 1998). Allelopathic chemicals released by plant roots, such as flavonoids and salicin, are known to induce catabolic enzymes in microorganisms—these enzymes also enhance the degradation of structurally similar pollutants through cometabolic processes (Wenzel 2009). However, this is not always the case, and a range of plant root extracts that enhanced growth and naphthalene degradation by the naphthalene degrader *Pseudomonas fluorescens* HK44 did not lead to increased specific expression of *nahG*, encoding naphthalene dioxygenase. The higher naphthalene degradation rates observed were due entirely to the growth stimulation provided by the root extracts (Kamath et al. 2004).

In addition to the nonspecific enhancement of PAH degradation described above, there may also be a specific three-way interaction between plants, microorganisms and PAHs, i.e. plants may utilize root colonizing microorganisms to protect themselves from PAH contamination, and may actively stimulate such organisms in the rhizosphere by manipulation of exudate flux and composition. Plants are known to be able to modify rhizosphere conditions depending on the environment in which they are growing. In the presence of a chemical stressor, plants may change the composition or the amount of root exudation (Walton et al. 1994), and these changes may be specific drivers in enhancing pollutant degradation, by altering the microbial community structure, stimulating the growth of microorganisms, or increasing microbial catabolic activities. In addition, specific bacteria may themselves alter the rhizosphere microbial community indirectly, by influencing plant root exudation flux

and composition (Matilla et al. 2010; Meharg and Killham 1995; Siciliano and Germida 1997)

In this study, we adopted a split-root model to examine the ability of a range of grass and legume species to influence the composition of the fungal and bacterial rhizosphere communities in response to PAH stress. Development of total microbial communities in PAH-contaminated and uncontaminated halves of the split-rhizospheres was studied and characterized. Significant differences in the plant response were seen for legumes and grasses, in two different soils, and bacterial genera that appeared to be responding to plant PAH-stress signalling were identified. The results suggest that legume species respond to PAH-contamination stress in a systemic manner, to help determine the rhizosphere community composition under these conditions.

Materials and methods

Soils and media

Sandy loam agricultural topsoils were purchased from Lindow Turf (Cheshire, UK) or collected from the University of Sydney's Lansdowne Farm (Cobbitty, NSW, Australia; 34°01′19″S 150°40′38″E). They were sieved before use (2 mm), and stored at 4°C in the dark. Characterization of critical soil parameters for the two soils, including mineral nutrient content, is given in Table 1.

Freshly PAH-contaminated soil was prepared by adding phenanthrene or pyrene dissolved in acetone to the sieved soil (Cunliffe and Kertesz 2006). PAH/acetone solution was first added to 10% of the soil to give a ten-fold contaminated soil and the solvent was allowed to evaporate in a fume hood. This soil was mixed with the remaining 90% of the soil to give final PAH concentrations of 1,000 μg g^{-1} soil dry weight (phenanthrene) or 500 μg g^{-1} soil dry weight (pyrene). The water content of the soil was adjusted to 35% of water-holding capacity (WHC) before use by addition of sterile tap water. Where required, 1/4 Hoagland's solution was used for plant nutrition, containing 1 mM KNO_3, 1 mM $NaNO_3$, 0.75 mM $MgSO_4$, 0.5 mM K_2SO_4, 2.1 mM $CaCl_2$, 0.307 mM NaH_2PO_4, 0.026 mM Na_2HPO_4, 8.6 μM $MnSO_4$, 2 μM $ZnSO_4$, 1 μM $CuSO_4$, 50 μM H_3BO_3, and 10 μM ferric citrate.

Table 1 Soil properties

	Lindow soil (UK)	Lansdowne farm soil (Aus)
pH	6.6	7.5
Texture	Sandy loam	Sandy loam
Total nitrogen (g/100 g soil)	0.09	0.14
Extractable phosphorus (mg/l)	25.0	44.5
Extractable potassium (mg/l)	72.3	273.7
Extractable magnesium (mg/l)	62.1	315.9
Organic carbon (g/100 g)	1.08	1.56

Plant growth conditions and construction of the split-root system

White clover (*Trifolium repens*), ryegrass (*Lolium perenne*), red fescue (*Festuca rubra*), Yorkshire fog (*Holcus lanatus*), subterranean clover (*Trifolium subterraneum*), red lentil (*Lens culinaris* var. *microsperma*), and chickpea (*Cicer arietinum*) seeds were purchased from Emorsgate Seeds (Norfolk, UK) or Heritage Seeds (Mulgrave, Vic., Australia). Before germination, seeds were surface sterilized by washing with 70% ethanol for 30 s, 20% (v/v) bleach for 5 min and rinsing 6–7 times with sterile water. Seeds were then soaked in sterile water for at least 2 h, and germinated on water agar (2% (w/v)) for 4 days at 25°C in the dark. The seedlings were then transplanted onto washed sand and watered with 1/4 Hoagland's solution for 10 to 30 days to allow proliferation of the extensive root system. Once the root system had grown sufficiently, the seedling was transferred into a small plastic pot (27×27×45 mm) in such a way that the root system extended from two holes (approx. 10×25 mm) at the bottom of the pot. The pot was then filled with sand and stood in 1/4 Hoagland's solution for 10 days to encourage further root proliferation. After 10 days, the split-root system was constructed by fixing the small pot containing the plant onto the middle of two larger pots (38×38×50 mm) (Fig. 1) with the roots extending into the pots on either side, which contained either PAH-contaminated soil or uncontaminated soil (Table 2). Split-root treatments were constructed in triplicate, watered in the bottom pots with autoclaved tap water, and cultivated for 30 days (clover, fescue and Yorkshire fog) or 21 days (all other plants) For experiments conducted in the UK, plants were maintained in a growth chamber (CLF Plant Climatics, Emersacker, Germany) under controlled conditions (16 h light/8 h dark, 23°C,

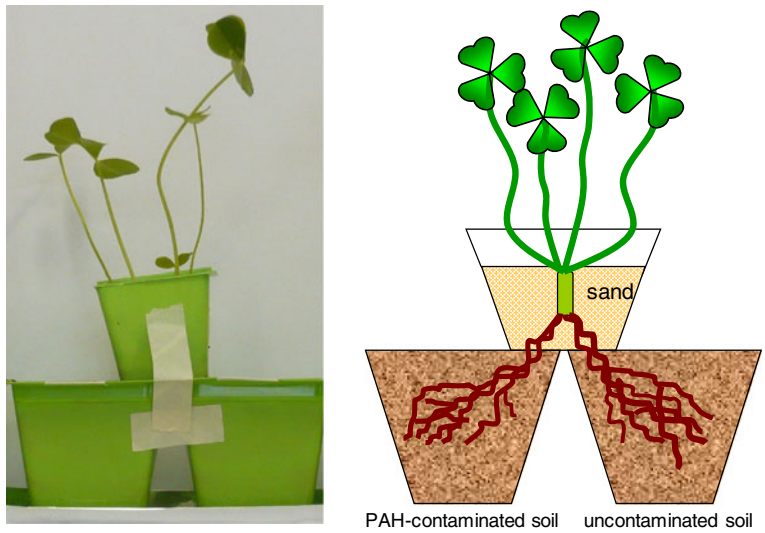

Fig. 1 Split-root model. The root system from a single plant growing in sand (top pot) was carefully divided into two, with one part of root system growing into the PAH-contaminated soil, and the other part growing into uncontaminated soil (bottom pots)

light intensity 1,250 μmol.m^{-2}.s^{-1}), while for experiments carried out in Australia, plants were grown in propagators (38 cm x 24 cm x 18 cm) in a green house with natural light conditions and the temperature maintained at 23°C. Rhizosphere samples were harvested by carefully extracting the roots from the bottom pots and removing excess bulk soil by gentle shaking, and were stored at −80°C for subsequent analysis.

The treatments were differentiated into heterogeneous treatments, in which the two half-rhizospheres were exposed to different conditions (designated *Het-Pyr* or *Het-Phe* for the respective contaminated side and *Het-C*$_{pyr}$ or *Het-C*$_{phe}$ for the control side), and homogeneous treatments, in which both sides of the plant experienced the same treatment, either PAH exposure, (*Hom-Pyr* or *Hom-Phe*) or uncontaminated soil (*Hom-C*) (Table 2).

DNA extraction and pyrosequencing analysis

DNA was extracted from the rhizosphere (roots and attached soil) using the FastDNA SPIN Kit for Soil (Qbiogene) or PowerSoil DNA Isolation Kit (Mobio) according to the manufacturer's protocols. Purified rhizosphere DNA was stored at −20°C. The dominant bacterial taxa in the rhizosphere communities were identified by tag-pyrosequencing of 16S rRNA gene amplification products obtained from triplicate samples. Initial PCR amplification was done using 63F (AGGCCTAACACATGCAAGTC) and 1087R (CTCGTTGCGGGACTTACCCC) primers in a total volume of 50 μl, containing 10 μl PCR Reaction Buffer A (5×, KAPA Biosystem), 200 μM dNTPs, 20 pmol of each primer, 5 μg BSA, 1 unit KAPA 2G Robust DNA polymerase (KAPA Biosystem), and 2 μl of tenfold diluted rhizosphere DNA as the template. After initial denaturation at 95°C for 5 min, the reactions were run for 30 cycles of 95°C for 45 s, 55°C for 45 s, and 72°C for 1 min with a final elongation step at 72°C for 5 min in an S1000 Thermal Cycler (Bio-Rad). PCR products were purified using ISOLATE PCR and Gel Kits (Bioline) and the DNA concentration was adjusted to 20 ng.μl^{-1}. The purified PCR products of the triplicate samples were then

Table 2 Split-root model rhizosphere sample labelling. Combination of bottom pots, with or without PAH, and the name of rhizosphere samples in each split-root treatment

Treatment	Combination of two bottom pots	Rhizosphere sample name
Heterogeneous split-root model	+ pyrene or phenanthrene	*Het-Pyr* or *Het-Phe*
	uncontaminated	*Het-C*$_{pyr}$ or *Het-C*$_{phe}$
Homogeneous split-root model (PAH-contaminated)	+ pyrene or phenanthrene	*Hom-Pyr* or *Hom-Phe*
Homogeneous split-root model (uncontaminated)	uncontaminated	*Hom-C*

pooled, and subjected to tag encoded-pyrosequencing analysis from the 63F primer, using Roche 454 FLX Titanium Technology (Research and Testing Laboratories, Lubbock, Texas). Sequences obtained from the FLX sequencing runs were filtered as previously described (Acosta-Martinez et al. 2008), to remove poor quality and short runs, and chimeras. Phylogenetic assignment was done using a custom database derived from the RDP-II database (Acosta-Martinez et al. 2008), with family, genus and species identities set at 89%, 94% and 96% identity, respectively.

Terminal restriction fragment length polymorphism (T-RFLP) analysis

T-RFLP analysis of rhizosphere bacterial and fungal communities was carried out by targeting 16S rRNA genes and internal transcribed spacer (ITS) genes respectively, using primers labelled at the 5′ end with 5-carboxyfluorescein (FAM) or hexachlorofluorescein (HEX). For bacterial communities, T-RFLP was performed with 63 F-FAM and 1087R-HEX primers (Singh et al. 2008), using the PCR program described above in a total volume of 50 μl, containing 5 μl PCR Reaction Buffer (10×, Bioline), 1.5 mM $MgCl_2$, 200 μM dNTPs, 20 pmol of each primer, 5 μg BSA, 1 unit BIOTAQ DNA polymerase (Bioline), and 2 μl of 10-fold diluted rhizosphere DNA as the template. Fungal community analysis was done with ITS5-FAM (GGAAGTAAAAGTCGTAACAAGG) and ITS4-HEX (TCCTCCGCTTATTGATATGC) primers (White et al. 1990), under the same conditions, but with a temperature of 54°C in the annealing step. The PCRs were carried out in a Whatman Biometra T1 thermocycler (Biometra). Four 50 μl reactions were prepared for each sample, and the combined product was ethanol-precipitated and redissolved at 300 ng DNA μl^{-1}. The purified PCR product was digested with *HhaI* restriction endonuclease (Fermentas) overnight at 37°C, and 0.5 μl of the digested DNA (75 ng) was mixed with 9.45 ml of HiDi formamide (Applied Biosystems) and 0.05 μl of GS500 LIZ size standard (Applied Biosystems). Capillary electrophoresis of the samples was carried out using an ABI 3730 automated DNA sequencer (Applied Biosystems) with two technical replicates for each sample (University of Manchester sequencing unit). Terminal restriction fragment (T-RF) size analysis was performed using GeneMapper 4.0 (Applied Biosystems) to define peak size, and the output was further analyzed with T-Align (http://inismor.ucd.ie/~talign/) to generate a consensus profile of the T-RF sizes between the technical duplicates and to compare this profile between the samples (Smith et al. 2005). Presence or absence of the T-RF was scored and used for the statistical analysis.

Denaturing gradient gel electrophoresis (DGGE) analysis

The V3 region of the bacterial 16S rRNA gene was amplified by PCR using GC-341F (CGCCCGCCGCG CGCGGCGGGCGGGGCGGGGGCACGGGGGG CCTACGGGAGGCAGCAG) and 518R (ATTACCG CGGCTGCTGG) primers (Muyzer et al. 1993). The PCR was carried out in a 50 μl reaction mixture consisting of 10 μl PCR Reaction Buffer A (5×, KAPA Biosystem), 200 μM dNTPs, 20 pmol of each primer, 1 unit KAPA 2 G Robust DNA polymerase (KAPA Biosystem), and 1 μl of 10-fold diluted rhizosphere DNA as the template. PCR amplification was carried out in an S1000 Thermal Cycler (Bio-Rad), and consisted of initial denaturation at 95°C for 5 min, followed by 10 cycles of 95°C for 30 s, 60°C (reducing by 1°C/cycle) for 30 s, 68°C for 30 s and a further 20 cycles of 95°C for 30 s, 50°C for 30 s, 68°C for 30 s with a final elongation step at 68°C for 5 min.

DGGE was performed with a DCode 16 cm×16 cm gel system (Bio-Rad) as described previously (Cunliffe and Kertesz 2006), with a denaturant gradient of 30–60%, poured with a gradient mixer. The gel was loaded (200 ng/sample) and electrophoresis was conducted at 60°C for a total of 1,008 V hours (constant voltage of 63 V for 16 h). After completion, gels were stained with 2 μl SYBR Gold stain (Invitrogen) in 20 ml 1xTAE for 30 min in the dark and then washed briefly with dH_2O. Gel image analysis was carried out with the Quantity One V4.6.9 software (Biorad).

Extraction of soil PAHs, and quantification by high performance liquid chromatography (HPLC)

Before extraction of PAHs from soil samples (1 g dry weight), an internal benzo[*ghi*]perylene standard (5 μL; saturated solution in acetonitrile) was added, in order to quantify extraction efficiency. The soil sample was then extracted twice in 6 ml of acetone/dichloromethane (1:1) in an ultrasonic water bath (Ultrawave Limited, Cardiff, UK) for 10 min, followed by centrifugation in

glass centrifuge tubes at 3,600 rpm for 10 min. The supernatants were combined and the solvents removed by air blow down at 60°C. The residue was then re-dissolved in 2 ml of acetonitrile by ultrasonication for 10 min, centrifuged at 13,200 rpm for 10 min and filtered (0.45 μm nylon syringe filter).

PAHs in the sample were separated by reversed-phase HPLC on a Supelcosil LC-PAH column (Supelco) with a Supelcosil LC-18 Supelguard guard column (Supelco), using a Dionex HPLC system and Chromeleon software (Dionex). The mobile phase was water with a methanol gradient (80 to 100%, vol/vol) and the flow rate was 1 ml min^{-1}. Pyrene, phenanthrene and benzo[*ghi*]perylene were detected spectrophotometrically at 254 nm with a Dionex PDA-100 Photodiode Array Detector (Dionex), or by fluorescence with an RF 2000 Fluorescence Detector (Dionex), using 260 nm excitation wavelength and 420 nm emission wavelength.

Statistical analysis

Presence or absence of T-RFs and DGGE bands were converted into binary matrices, and non-metric multidimensional scaling (NMDS) was performed using IBM SPSS Statistics 19 (IBM) with the Euclidean distance. NMDS results were further subjected to analysis of similarities (ANOSIM) with the Euclidean distance using Palaeontological Statistics (PAST) package to assess the similarities between the rhizosphere microbial communities.

Results

Impact of heterogeneous PAH contamination on the bacterial community structure of white clover rhizospheres

In order to examine indirect, plant-mediated effects of soil contamination on rhizosphere microbial communities, we constructed a split root experiment with white clover (*T. repens*) grown in Lansdowne Farm soil, and treated with either pyrene (500 μg/g soil) or phenanthrene (1,000 μg/g soil) under homogeneous or heterogeneous treatment conditions. Direct effects of contamination on the microbial community were investigated in the homogeneous split root systems, while system effects were studied in the heterogeneous systems, by assessing the rhizosphere microbial community structure of each half rhizosphere by DGGE (Fig. 2a and b). For homogeneous split-root systems, the bacterial communities present in the pyrene-contaminated and uncontaminated control rhizospheres (*Hom-Pyr* and *Hom-C*) after 30 days were significantly different from each other (p=0.0016) (Fig. 2a). This is an expected result, and the change in community structure over this time is likely to represent enrichment in the contaminated rhizosphere of microbial strains that are either pyrene-resistant or able to degrade the compound. However, when the plant was grown in the heterogeneous split-root system (one side contaminated with pyrene and the other side uncontaminated), the community structure of *Het-*C_{pyr} was significantly different from that of *Hom-C* (p=0.025), and the microbial communities in the two halves of the heterogeneous system were much more similar to each other than to the corresponding *Hom-C* and *Hom-Pyr* controls (Fig. 2a). This suggested that pyrene contamination of one half of the root system was able to influence microbial community development in the other half of the system. Using HPLC and fluorometric detection, we were unable to detect plant-mediated transfer of pyrene from the *Het-Pyr* rhizosphere into the *Het-*C_{pyr} rhizosphere, and this suggests that the effect must be an indirect one, with the white clover plant itself responding to pyrene-contamination stress in a systemic manner to influence the composition of the rhizosphere bacterial community.

By contrast, in split-root systems contaminated with phenanthrene, the rhizosphere bacterial communities were grouped entirely by the presence or absence of the phenanthrene, with *Hom-Phe* and *Het-Phe* showing significantly different communities from *Hom-C* and *Het-*C_{phe} (Fig. 2b). The indirect contamination effect that was seen in the pyrene split-root systems (i.e. *Het-*C_{pyr} significantly different from *Hom-C*) was not observed in the phenanthrene split-root system. At the level of plant yield, white clover also displayed a much greater level of growth inhibition in response to pyrene contamination than for phenanthrene contamination, with 51% (p=0.038) and 28% (p=0.226) reduction in the shoot dry weights respectively (n=5), suggesting that the plants were less susceptible to PAH stress from phenanthrene. This may explain the absence of the indirect contamination effect on microbial communities in phenanthrene-treated clover plants.

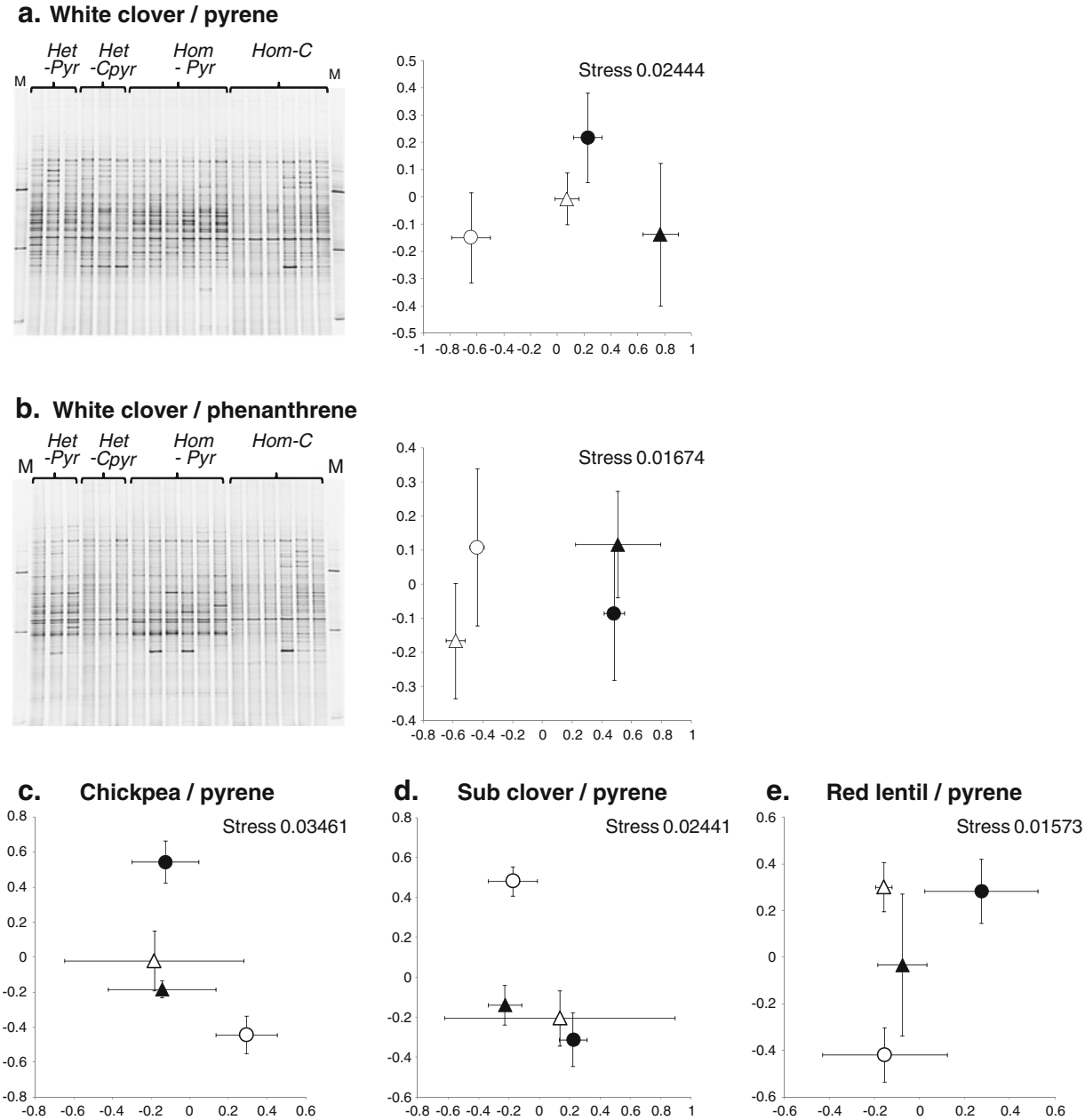

Fig. 2 16S PCR-DGGE profiles and NMDS ordination plots of legume rhizosphere bacterial communities. The split root systems were done in Lansdowne farm soil, and contained **a** white clover with pyrene; **b** white clover with phenanthrene; **c** chickpea with pyrene; **d** subterranean clover with pyrene; **e** red lentil with pyrene contamination. Initial PAH concentrations were 500 μg pyrene/g soil or 1,000 μg phenanthrene/g soil. Rhizosphere bacterial community structures were analyzed with 16S PCR-DGGE; M - marker. Closed triangle, *Het-Pyr* or *Het-Phe*; open triangle, *Het-*C_{pyr} or *Het-*C_{phe}, closed circle, *Hom-Pyr* or *Hom-Phe*; open circle, *Hom-C*. Data represent mean values of ordination coordinates±SE; n=3–6

Indirect rhizosphere response to PAH-contamination stress in other legume species

In order to examine the plant specificity of the effect observed in white clover, split-root experiments were carried out with a range of legume species under similar conditions to those above. Chickpea, subterranean clover and red lentil were cultivated using the split-root system in Lansdowne Farm soil with or without 500 μg pyrene per gram soil. The plants were

harvested at 21 days, when they reached maturity. DNA was extracted from the half rhizospheres and DGGE analyses were conducted to assess the rhizosphere bacterial community structures.

NMDS based on the DGGE banding pattern showed that in chickpea and subterranean clover, indirect PAH contamination affected the bacterial community structure of the *Het-C$_{pyr}$* rhizospheres (Fig. 2c & d), since the *Het-C$_{pyr}$* communities were significantly different from those of *Hom-C* (p=0.0364 and 0.0106 respectively, for the two species). This confirms the effect that was seen in the white clover pyrene split-root system (Fig. 2a). In the red lentil split-root system, a separation of the bacterial community structure between *Het-C$_{pyr}$* and *Hom-C* can also be seen in the NMDS plot (Fig. 2e), but is not statistically significant (p=0.1084) (Fig. 2e). Growth of both chickpea and subterranean clover was significantly affected by the pyrene contamination, with 27% (p=0.005, n=5) and 31% (p=0.001, n=5) reduction in their shoot dry weights respectively. Red lentil showed 17% reduction in its shoot dry weight in response to pyrene contamination, but this was not statistically significant (p=0.171, n=5). As for white clover, the indirect PAH contamination effect on the rhizosphere microbes is most apparent when the plant also shows a growth phenotype caused by the level of PAH applied.

Pyrene degradation in the legume rhizospheres was also examined, using HPLC with fluorometric detection. In the pyrene-contaminated bulk soil, pyrene remained stable over the time period, and after 30 days of soil incubation only 4.9% had been degraded. In the rhizospheres of chickpea, subterranean clover and red lentil, by contrast, 16.1%, 22.1% and 32.9% of pyrene, respectively, had been degraded after 21 days of incubation.

Specificity of the indirect PAH rhizosphere response to soil source

Plant-microbe interactions are often decisively influenced by the particular soil in which the plants are grown. To test whether the indirect PAH response observed above was a general phenomenon, we performed the white clover split-root experiment in a second soil (Lindow soil), which was obtained from Lindow Turf, Cheshire, UK. The plants were cultivated in split-root systems as before, with pyrene contamination (500 μg/g soil) where appropriate. Rhizospheres were harvested after 30 days, and total rhizosphere DNA was extracted. The robustness of the analytical method used was also tested in this case, by conducting the community analysis with T-RFLP rather than DGGE, followed by a binary analysis of the T-RFs present, and ordination analysis as before with NMDS and ANOSIM.

In the split-root system set up with Lindow soil, the indirect contamination effect on the rhizosphere bacterial community was once again evident (Fig. 3a, 16S; *Het-C$_{pyr}$* significantly different from *Hom-C;* p=0.0081). This confirmed the result seen after growth in Lansdowne Farm soil and demonstrated that the white clover response to pyrene stress and the subsequent effect on the rhizosphere bacterial community is not specific to the soil tested. The fungal community in white clover half-rhizospheres was also analyzed, using T-RFLP targeting the ITS region. Ordination analysis (NMDS/ANOSIM) showed an apparent separation between the *Het-C$_{pyr}$* and *Hom-C* fungal communities (Fig. 3a, ITS), just as it had for the corresponding bacterial communities (Fig. 3a, 16S), but the difference in the fungal community structures was not statistically-significant (p=0.0911).

Heterogeneous PAH contamination does not control the rhizosphere community structure of grass species

In order to test whether the indirect PAH contamination effect observed above for legumes is also seen in other plants, we conducted split-root experiments on three grass species, ryegrass (*Lolium perenne*), red fescue (*Festuca rubra*) and Yorkshire fog (*Holcus lanatus*). Initially, we tested ryegrass for the indirect PAH contamination effect by cultivating it in the split-root system using Lindow soil with pyrene contamination (500 μg/g soil) for 21 days. T-RFLP analysis of the ryegrass rhizosphere bacterial communities showed that the community structures were not affected by pyrene treatment of the other half of the plant (*Het-C$_{pyr}$* was not significantly different from *Hom-C*, Fig. 3b). At this concentration of pyrene (500 μg/g soil), ryegrass growth showed a 23% reduction in the shoot dry weight, but it was not significantly inhibited (p=0.084, n=5), and we postulated that the absence of an indirect PAH contamination effect might be due to insufficient inhibition of ryegrass by pyrene at this level.

In order to find a PAH concentration that would affect the plant growth of grass species, red fescue and

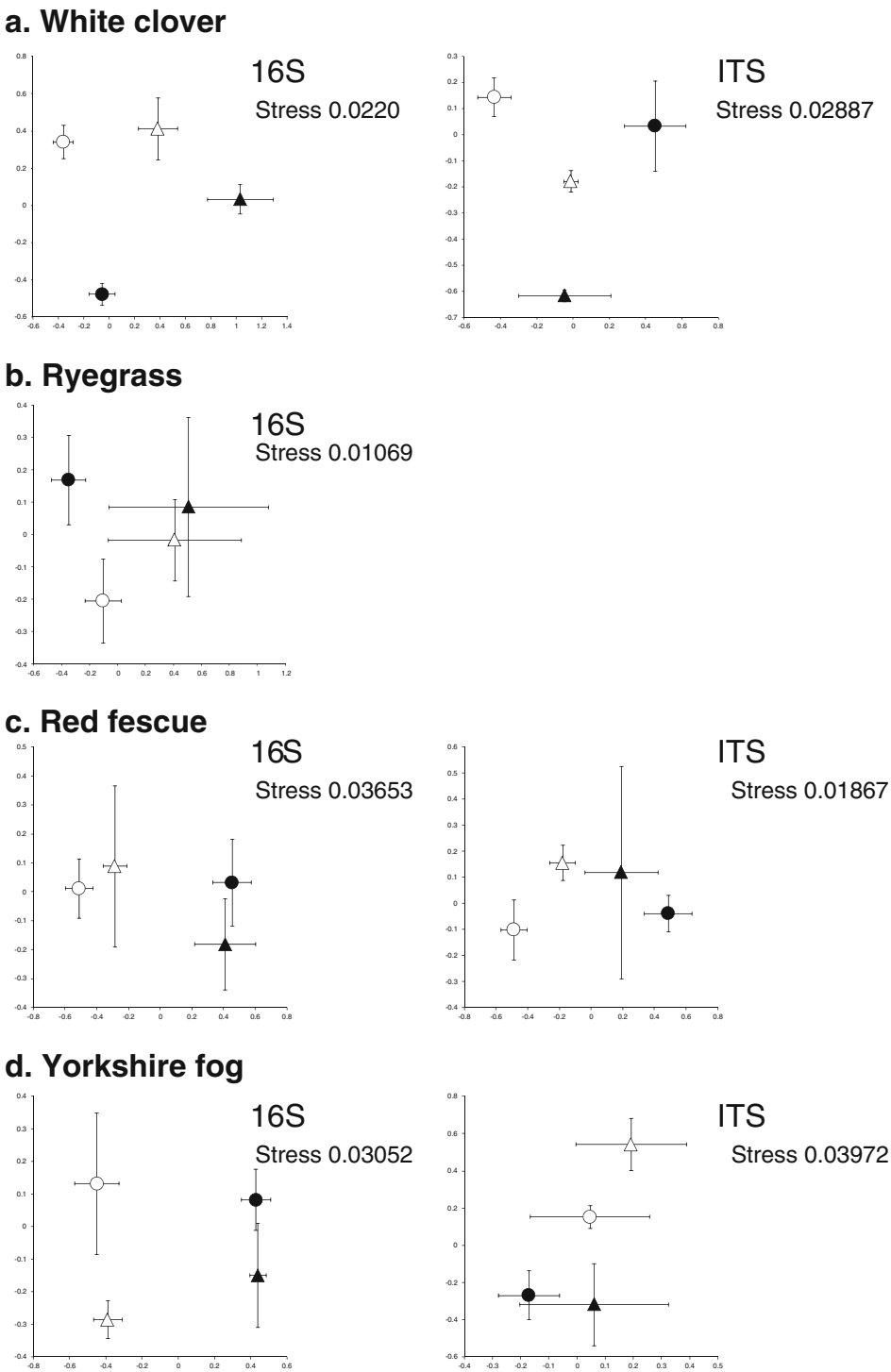

Fig. 3 NMDS ordination plots of the rhizosphere bacterial (16S) and fungal (ITS) community structures revealed by T-RFLP. The split root systems were done in Lindow soil, and contained **a** white clover with pyrene; **b** ryegrass with pyrene; **c** red fescue with phenanthrene; **d** Yorkshire fog with phenanthrene contamination. Closed triangle, *Het-Pyr* or *Het-Phe*; open triangle, *Het-*C_{pyr} or *Het-*C_{phe}, closed circle, *Hom-Pyr* or *Hom-Phe*; open circle, *Hom-C*. Data are mean values of ordination coordinates±SE; n=3–6

Yorkshire fog were initially examined by growing in soil contaminated by pyrene (500 μg/g) or phenanthrene (1,000 μg/g soil). Red fescue and Yorkshire fog growth was significantly affected by the pyrene contamination, which resulted in 34% (p=0.007, n=5) and 37% (p=0.016, n=5) reduction in their shoot dry weights respectively. However, phenanthrene contamination showed a greater effect on the plant growth, reducing red fescue shoot dry weight by 56% (p=0.001, n=5) and Yorkshire fog shoot dry weight by 47% (p=0.000, n=5). Therefore, we conducted split-root experiments on red fescue and Yorkshire fog in the Lindow soil with 1,000 μg/g soil phenanthrene contamination. The plants were cultivated for 30 days and the rhizosphere bacterial and fungal communities were analyzed with T-RFLP. Although growth of both grass species was significantly affected by the phenanthrene contamination, ordination analysis (NMDS/ANOSIM) showed that bacterial and fungal community structures could only be grouped by the presence or absence of phenanthrene (*Hom-Phe* and *Het-Phe* were significantly different from *Hom-C* and *Het-C*$_{phe}$, p<0.05) (Fig. 3c & d). The indirect PAH contamination effect which was seen in white clover (*Het-C*$_{pyr}$ significantly different from *Hom-C*) was not observed in either bacterial or fungal communities in grass rhizospheres. This result indicates that the indirect PAH contamination effect on the rhizosphere community via the plant is not observed in all plants, and more work is required to delineate the range of this phenomenon.

Tag sequencing analysis reveals bacterial groups which respond directly and indirectly to pyrene contamination in white clover rhizosphere

In order to define which bacterial groups respond to pyrene contamination directly, and which respond indirectly, 16S tag pyrosequencing was carried out on the white clover split-root rhizosphere samples grown in the Lindow soil. A total of 9,052 valid sequences were obtained from *Het-Pyr*, *Het-C*$_{pyr}$, *Hom-Pyr* and *Hom-C* rhizosphere samples. The most dominant bacterial group in all rhizospheres was *Proteobacteria* (*Alpha, Beta, Gamma and Deltaproteobacteria*), which represented 55–70% of the total bacterial community. Increased *Actinobacteria* and *Verrucomicrobia* populations were observed in *Het-Pyr* and *Hom-Pyr*, suggesting that these two phyla respond directly to the pyrene contamination. Meanwhile, a decrease in *Gammaproteobacteria* was observed in *Het-Pyr* and *Hom-Pyr* (Fig. 4), suggesting that members of this taxon have higher sensitivity to pyrene contamination than other phyla. At the genus level, *Denitratisoma* genotypes were enriched in the presence of pyrene, and made up the most abundant genus in *Het-Pyr* and *Hom-Pyr*. *Bacillus* appeared to respond indirectly to pyrene stress via the plant, as the proportion of *Bacillus* sequences increased from 3% in *Hom-C* to 9% in *Het-C*$_{pyr}$ (Table 3), and members of this genus may be responding to specific metabolites released by the plant when subjected to pyrene stress.

Discussion

Enhanced microbial PAH degradation in plant rhizospheres has been demonstrated before, but the complex interactions between the plant and the root-colonizing microbes that occur during the rhizoremediation process still remain unclear. A three-way interaction between plant, microbe and pollutant

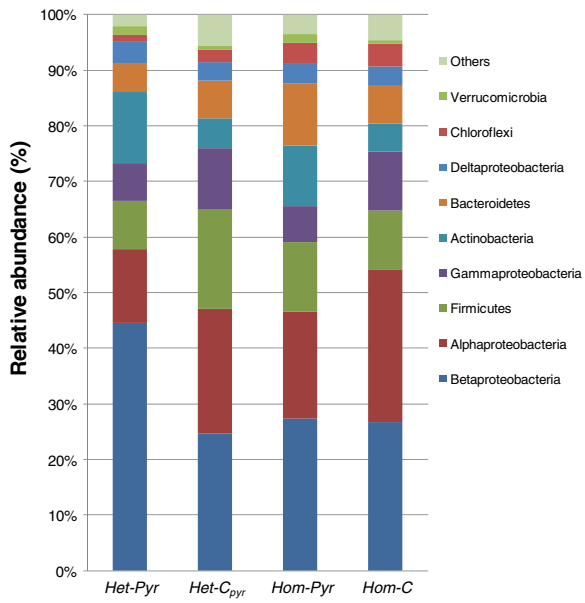

Fig. 4 Relative abundance of bacterial phyla in the white clover split-root rhizospheres revealed by tag pyrosequencing of the 16S rRNA gene amplicon. The split-root models were cultivated in the Lindow soil with pyrene contamination. The phylum *Proteobacteria* was further sub classified into classes

Table 3 Dominant bacterial genera found in the white clover split-root rhizospheres. The split-root models were cultivated in the Lindow soil with pyrene contamination as appropriate. The dominant genera were identified by tag-pyrosequencing of the 16S rRNA gene in DNA isolated directly from the rhizosphere soil. The fifteen dominant genera are listed for each sample

	Het-Pyr		*Het-C_{pyr}*		*Hom-Pyr*		*Hom-C*	
rank	Genus	%	Genus	%	Genus	%	Genus	%
1	*Denitratisoma*	23.66	*Bacillus*	9.14	*Denitratisoma*	8.33	*Methylovorus*	6.40
2	*Mycobacterium*	9.94	*Paenibacillus*	7.21	*Paenibacillus*	7.07	*Paenibacillus*	5.31
3	*Paenibacillus*	4.46	*Methylovorus*	3.76	*Hydrogenophaga*	5.72	*Bacillus*	3.41
4	*Cupriavidus*	3.59	*Hydrogenophaga*	2.34	*Niastella*	3.29	*Levilinea*	2.36
5	*Rhodoferax*	3.33	*Lysobacter*	2.30	*Rubrobacter*	2.52	*Ideonella*	2.36
6	*Bacillus*	3.20	*Flavisolibacter*	2.07	*Polyangium*	2.42	*Rhizobium*	2.13
7	*Polyangium*	2.94	*Gemmatimonas*	2.07	*Hymenobacter*	2.42	*Afipia*	1.80
8	*Hydrogenophaga*	2.79	*Pedomicrobium*	1.93	*Methylovorus*	2.33	*Hydrogenophaga*	1.76
9	*Methylovorus*	2.06	*Byssovorax*	1.84	*Candidatus Monilibacter*	2.33	*Pedomicrobium*	1.65
10	*Afipia*	2.06	*Novosphingobium*	1.70	*Burkholderia*	2.13	*Methylibium*	1.65
11	*Niastella*	1.65	*Nocardioides*	1.65	*Sediminibacterium*	2.03	*Ramlibacter*	1.53
12	*Chthoniobacter*	1.03	*Methylibium*	1.65	*Bacillus*	1.84	*Polyangium*	1.50
13	*Ramlibacter*	1.01	*Ktedonobacter*	1.56	*Novosphingobium*	1.74	*Caulobacter*	1.46
14	*Schlegelella*	0.98	*Devosia*	1.47	*Mycobacterium*	1.65	*Devosia*	1.46
15	*Pedomicrobium*	0.93	*Ideonella*	1.47	*Pseudomonas*	1.55	*Byssovorax*	1.42

has been suggested, in which a plant that detects pollution in the soil may respond by changing the rate or composition of root exudation, thereby stimulating pollutant degradation by the rhizosphere microbes (Walton et al. 1994) and possibly recruiting specific pollutant degraders to its rhizosphere (Siciliano and Germida 1998). In this study, we show that legumes demonstrate considerable influence over the microbial community composition in their rhizospheres in response to PAH contamination, while this effect was much less pronounced for the three grasses studied.

The concept of the split-root system has been used in a wide range of studies, mainly investigating plant responses to environmental biotic and abiotic stressors, and for elucidating shoot-root signalling in nutrient uptake and transport. For instance, split-root systems have been applied to study the rhizodeposition of legume-derived N into the soil (Schmidtke 2005), and the suppression of nodule development on soybeans (Kosslak and Bohlool 1984). However, so far there are only few published studies that apply split-root systems for studying rhizoremediation of contaminated soils. Langer et al. (2010) used a split-root system to study the effect of heterogeneous hydrocarbon pollution and arbuscular mycorrhizal fungi (AMF) colonization on bush bean growth and root morphology, by contaminating one side of the split-root system with crude oil, or inoculating it with AMF. Heterogeneous pollutant exposure or AMF colonization caused different plant responses to homogeneous conditions (polluted or non-polluted). In the heterogeneous polluted condition, increases in root biomass, root length and foliar phosphorus content were observed compared to the homogeneous conditions.

In this study, we assessed the shift in the rhizosphere microbial population in various plant species during rhizoremediation of PAH-contaminated soils. Our findings suggest that legume species specifically influenced their rhizosphere microbial composition in response to PAH stress. When white clover, chickpea and subterranean clover were cultivated with pyrene contamination in one side of the split-root system, the microbial population in the counterpart uncontaminated rhizosphere (*Het-C_{pyr}*) was significantly different from that observed when the plants were grown in homogeneous uncontaminated control conditions (*Hom-C*) (Fig. 2a, c & d, Fig. 3a). In principle, this

could be explained by a transfer of contaminant from one side of the plant to the other, either through capillary action or plant-mediated transfer. *Trifolium* is indeed known to be able to uptake PAH to their roots, but translocation to the shoots is very restricted and the concentration of PAH in the shoots is 100 times lower than in the roots (Gao et al. 2008). A similar effect also occurs in wheat, with no phenanthrene transported to the shoots from the roots (Li et al. 2001). In our heterogeneous contaminated split-root system we were unable to detect PAH in the uncontaminated rhizosphere, and it is therefore unlikely that the change in microbial community was due to direct PAH transfer from one side of the plant to the other in the split-root system. A plausible alternative mechanism is that leguminous plants change their exudation, qualitatively or quantitatively, in response to the PAH stress, and that this specifically influences the rhizosphere microbial community.

Plants are known to change their root exudation in response to various environmental stresses. A study on the effect of phenanthrene contamination on sorghum root exudation showed that when the plant was grown in a high concentration of phenanthrene (100 mg/kg), the total amount of exuded compounds decreased by 78%, due to phenanthrene damaging the root system. The composition of the root exudates was, however, qualitatively the same as for the control plant (Muratova et al. 2009a), though the activities of three oxidoreductases (oxidase, peroxidase, and tyrosinase) were increased in the presence of phenanthrene (Muratova et al. 2009b). The composition and flux of exudate is also strongly dependent on the presence of microbes in the rhizosphere (Meharg and Killham 1995; Siciliano and Germida 1997), and these microbe-induced changes in exudate quality may also directly affect pollutant degradation in the rhizosphere. Exudates from *Trifolium* plants have been shown to enhance rhizosphere degradation of 2,4-dichlorophenoxyacetic acid (2,4-D), but only when the exudates were harvested from plants grown in the presence of microorganisms (Shaw and Burns 2005a). This stimulatory effect observed for *Trifolium* exudates may be related to the indirect PAH-induced community shift we report in this paper, since it was very much stronger for legumes than for other plant species (Shaw and Burns 2004). The effect was clearly linked to an exudate or root-debris associated factor and the authors proposed that flavonoids might play an integral role, but this could not be directly confirmed. In contrast to the results reported here, the increase in 2,4-D degradation varied greatly between soil types (Shaw and Burns 2005b), and no change in the diversity of 2,4-D degrading rhizosphere bacteria was observed, at least at the level of the *tfdA* gene (Shaw and Burns 2004).

Plant root exudates can also modify microbial community composition in PAH-contaminated soils. When ryegrass root exudates were added to a phenanthrene-contaminated soil, the population of Gram negative bacteria possessing PAH ring hydroxylating dioxygenase genes was significantly increased compared to the same contaminated soil without addition of exudates, and the diversity of the genes also differed between the exudate-treated and untreated soils (Cébron et al. 2011). Similarly, the diversity of aromatic ring-cleavage genes in PAH-contaminated birch rhizospheres was double that found in the surrounding PAH-contaminated bulk soils (Sipilä et al. 2008).

Plants only show their responses to the contaminant when they are exposed to levels above a "threshold concentration" of the contaminant (Muratova et al. 2009a). In this study, we confirmed that only legume species whose growth was inhibited by the PAH concentration tested showed responses to the PAH contamination by modifying their rhizosphere microbial populations. This response was clearly seen in the significant differences observed between *Hom-C* microbial communities and *Het-*C_{pyr} communities in the heterogeneous split-root systems, which suggests that the plant senses PAH contamination at *Het-Pyr*, and releases stress response signals to modify the microbial community structure of *Het-*C_{pyr} even when there is no PAH present there. This result provides clear experimental evidence for the importance of a three-way plant-microbe-pollutant interaction during the rhizoremediation process, as suggested by Walton et al. (1994).

Analysis of the bacterial communities in the white clover rhizospheres by tag-pyrosequencing (Fig. 4) identified several groups of bacteria that are directly enhanced by pyrene contamination, and others that are indirectly enhanced by pyrene contamination via the plant. In the pyrene-contaminated rhizospheres, *Het-Pyr* and *Hom-Pyr*, increased *Actinobacteria* and *Verrucomicrobia* populations were observed (Fig. 4), indicating that these two phyla were benefiting from

pyrene or pyrene degradation products. It is not surprising that *Actinobacteria* were enriched in the presence of pyrene since many *Mycobacterium* species are well known PAH degraders, and also colonize plant roots (Child et al. 2007). *Mycobacterium* was one of the 15 commonest genera observed in *Het-Pyr* and *Hom-Pyr* rhizospheres, but it was not found in the uncontaminated *Het-*C_{pyr} and *Hom-C* rhizospheres (Table 3). *Verrucomicrobia* are widely distributed in soil environments, making up 1–10% of bacterial 16S rRNA in soils, but only very few of these organisms have been isolated (Sangwan et al. 2005). Their ecological roles remain unclear, but it has previously been reported that they are negatively affected by PAH contamination and do not survive in PAH-contaminated soil (Yrjälä et al. 2010). This is contrary to our finding that *Verrucomicrobia* were stimulated in the presence of pyrene, and also to another study which reported that PAH-contaminated soil planted with alfalfa specifically favoured the development of *Verrucomicrobia* and *Actinobacteria* (Cébron et al. 2009). This may suggest that although *Verrucomicrobia* are inhibited by PAH contamination in the bulk soil, they are stimulated by compounds that the plant releases in response to PAH stress. This plant response is presumably local, and not systemic, since we did not observe stimulation of *Verrucomicrobia* in the *Het-*C_{pyr} rhizosphere. In the pyrene-contaminated rhizospheres (*Het-Pyr* and *Hom-Pyr*), the bacterial population was dominated by the *Denitratisoma* genus (Table 3). This genus was first reported in 2006 as the new species *Denitratisoma oestradiolicum*, a denitrifying organism related to *Azoarcus* and *Thauera* that was isolated from activated sludge of a wastewater treatment plant with 17-β-estradiol as the sole carbon source (Fahrbach et al. 2006). Little is yet known about this genus, but a related strain, with 95% 16SrRNA gene sequence identity to *Denitratisoma oestradiolicum*, was shown to utilize *p*-xylene as the sole carbon source under denitrifying conditions (Rotaru et al. 2010). Since the genus *Denitratisoma* was considerably enriched in our heterogeneously contaminated rhizospheres in the presence of pyrene (Table 3), this may suggest a potential of this genus for rhizoremediation applications.

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