

Specific influence of white clover on the rhizosphere microbial community in response to polycyclic aromatic hydrocarbon (PAH) contamination

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Abstract

Background and aims Legumes respond to PAH-contamination in a systemic manner and influence the overall rhizosphere microbial community structure, but the effect on the functional microbial community is unknown. In this study, plant-mediated PAH effects on specific bacterial taxa and the PAH-degraders in the rhizosphere were examined.

Methods White clover was cultivated using a split-root system, with one side exposed to phenanthrene or pyrene, and the other side uncontaminated. Rhizosphere microbial diversity and activity were assessed with DGGE and qPCR, and changes in the root exudation were analyzed with GC-MS and HPLC.

Results PAH contamination of one side of the rhizosphere significantly influenced the community structure of *Alphaproteobacteria*, *Betaproteobacteria*,

Gammaproteobacteria, *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Verrucomicrobia* in the uncontaminated side of the rhizosphere. This indirect PAH-effect also influenced the diversity of bacterial PAH dioxygenase genes present, though the expression levels of these genes was not affected. No significant difference in the root exudation of general metabolites (amino acids, organic acids, sugars and sugar alcohols) and a flavonoid was observed.

Conclusions In response to PAH-stress, white clover specifically influenced the diversity of the PAH-degrading community in its rhizosphere, but the abundance and activity of these PAH-degraders was not enhanced by the indirect PAH-effect. The plant-mediated response therefore does not appear to be directed towards enhanced removal of PAH for plant protection.

Keywords Polycyclic aromatic hydrocarbon · Rhizoremediation · Rhizosphere · Clover · DGGE · Microbial community · PAH dioxygenase · Root exudates

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Introduction

The rhizosphere interaction between plants and root-colonizing microbial communities is complex and has evolved to benefit both organisms (Anderson et al. 1993). Microbes benefit from the plant supply of sugars, organic acids and amino acids in root exudates, which support their growth and metabolic activity (Brimecombe et al. 2007). On the other hand, plant growth is stimulated by the rhizosphere population, since the rhizosphere

microbial communities enhance water and nutrient uptake, promote root growth, and are critical for nitrogen fixation and mobilization of mineral nutrients (Pilon-Smits 2005). Plants are also protected from hazardous organic contaminants such as polycyclic aromatic hydrocarbons (PAHs) by microbes which are able to degrade those compounds, in a process known as rhizoremediation (Kuiper et al. 2004).

PAHs are widely distributed in the environment, especially in urban and industrialized areas as they are mostly generated by anthropogenic activities (Wilson and Jones 1993). Some PAHs are known to possess mutagenic and carcinogenic properties, and they persist in the environment because of their low water solubility (Cerniglia 1992). Rhizoremediation is a promising technique to clean up PAH-contaminated soils, as higher rates of PAH-degradation are often observed in plant rhizospheres than are found in non-rhizosphere soil. This is primarily due to general enrichment of microorganisms through the rhizosphere effect (Corgié et al. 2003; Johnson et al. 2005). However, the composition of the rhizosphere community is also an important factor for successful rhizoremediation. Plants that can selectively enrich PAH-degraders in their rhizospheres perform better in enhancing degradation of contaminants (Siciliano et al. 2003).

The presence of plants has long been recognized to have a major selective influence on the diversity of soil microbial communities (Singh et al. 2007). Different studies have variously found the main drivers determining microbial community structure to be plant species or soil type (reviewed in Berg & Smalla (2009)), environmental influences such as management practices (Millard and Singh 2010; Sudini et al. 2011; Xuan et al. 2012) and the total plant community (Lamb et al. 2011). Unplanted Scottish grassland soils, for example, were dominated by *Bacilli*, *Clostridia* and *Actinobacteria*, but when planted with grasses *Lolium perenne*, *Anthoxanthum odoratum* or *Agrostis capillaries*, the population shifted to be mainly *Acidobacteria* and *Proteobacteria* (Singh et al. 2007). When less closely-related plants are studied, clear plant-specific differences in the rhizosphere community are seen (Marschner et al. 2004).

Contaminants in soils also have a considerable influence on the rhizosphere microbial community. PAH contamination significantly affects the microbial community structures in rhizospheres of grass and legume

(Kawasaki et al. 2012). and in important horticultural species such as pumpkin, zucchini, and lettuce (Pritchina et al. 2011). For legumes, the rhizosphere microbial community was both directly affected by the PAH contamination (e.g. enrichment of PAH-degrading organisms), and also influenced indirectly by the plant's response to the PAH contamination (Kawasaki et al. 2012). The observation of this indirect, plant-mediated effect suggests that legumes may influence both the rhizosphere diversity and its function in response to PAH-stress, perhaps in order to protect the plant by increasing PAH-degradation around the roots (Kawasaki et al. 2012). Enrichment of bacteria possessing aromatic catabolic genes in plant rhizospheres has been reported in several studies (Cébron et al. 2009; Siciliano et al. 2001; Siciliano et al. 2003). and this enrichment and stimulation of specific catabolic genotypes in the rhizosphere has been linked to changing root exudation patterns (Jha et al. 2015; Walton et al. 1994). However, it is not yet clear whether enrichment of these bacteria by plants is merely due to the rhizosphere effect, or whether plants specifically recruit and stimulate these communities in response to the pollutant stress.

In this study, we used a previously developed split-root system (Kawasaki et al. 2012) to examine how white clover (*Trifolium repens*) influences the functional rhizosphere community in response to PAH-stress. By exposing the plant roots in a heterogeneous system (one half of the rhizosphere contaminated with PAH, the other half uncontaminated), the split-root system enables us to study how the plant responds to the PAH contamination to affect the rhizosphere microbes without the direct presence of PAH. Development and activity of PAH-degraders and specific bacterial taxa in the rhizosphere were monitored, and we also examined how these responded to changes in plant root exudation (major plant metabolites and a flavonoid). White clover responded to PAH contamination by modifying the diversity of all rhizosphere bacterial taxa tested, and more importantly, the diversity of rhizosphere bacteria harbouring PAH-catabolic genes was also influenced by the plant PAH-stress response. However, this indirect PAH-effect did not enhance expression of these PAH-catabolic genes, and was not driven by the exudation of major plant metabolites (amino acids, organic acids, sugars and sugar alcohols) nor a flavonoid (7,4'-dihydroxy flavone).

Materials and methods

Soil, media and bacterial growth conditions

Sandy loam agricultural topsoil was collected from the University of Sydney's Lansdowne Farm (soil details previously published (Kawasaki et al. 2012)). PAH-contaminated soil was prepared by adding phenanthrene or pyrene dissolved in acetone to 10 % of the soil (10 ml acetone/100 g soil), and the solvent was allowed to evaporate. This soil was then mixed with the residual 90 % of the soil to give final concentrations of 1000 or 500 µg/g dry soil for phenanthrene or pyrene respectively (Kawasaki et al. 2012). Control uncontaminated soil was acetone-treated in the same manner, without addition of PAH.

For isolation and cultivation of rhizobia, yeast mannilol agar (YMA) [per litre - 1 g yeast extract, 10 g mannitol, 0.5 g K₂HPO₄, 0.2 g MgSO₄, 0.1 g NaCl, 1 g CaCO₃, and 1.5 % agar] and glucose broth [per litre - 0.75 g K₂HPO₄, 0.4 g MgSO₄·7H₂O, 5.13 g glucose, and 3 g yeast extract] were used. Susceptibility of rhizobial nodule isolates to pyrene was tested in pyrene-saturated glucose broth, by measuring growth of the isolate in 200 µl cultures in a 96-well microplate, using a Synergy H1 microplate reader (BioTek Instruments). The microplate was incubated at 25 °C with orbital shaking at medium speed, and OD₆₀₀ was measured every 10 min for 72 h.

Plant growth conditions

White clover (*Trifolium repens*) seeds were purchased from Heritage Seeds (Mulgrave, Australia), and split-root systems were set up as previously described with phenanthrene or pyrene contamination (1000 or 500 µg/g dry soil, respectively) (Kawasaki et al. 2012). Briefly, the white clover root system was split into two and allowed to grow out into two separated pots containing PAH-contaminated or uncontaminated soil. Three types of split-root treatments were prepared. These included (a) a heterogeneous split-root system with one side contaminated with pyrene or phenanthrene (designated *Het-Pyr* or *Het-Phe*), and the other side uncontaminated (designated *Het-C_{pyr}* or *Het-C_{phe}* for the respective uncontaminated control pots), (b) a homogeneous, PAH-contaminated split-root system (both sides contaminated with PAH (*Hom-Pyr* or *Hom-Phe*)), and (c) a homogeneous, control split-root system (both sides

uncontaminated (*Hom-C*)) (Table 1). The split-root systems were prepared in 3–5 replicates and cultivated for 30 days in a greenhouse under natural light conditions, with the temperature maintained at 23 °C. Rhizosphere samples were harvested after 30 days of cultivation and stored at –80 °C for subsequent analysis.

Measurement of nodulation, and isolation and characterization of rhizobia

Roots were washed carefully with tap water and nodules were excised using a scalpel. Total nodule and root weights were determined after drying (50 °C, overnight). For rhizobia isolation, fresh nodules were surface-sterilized by washing with 70 % ethanol for 30 s, 20 % (v/v) bleach for 5 min and then rinsed 6–7 times with sterile water. Nodules were crushed, and the nodule contents were streaked onto YMA plates. The resulting colonies were analyzed by 16S rRNA gene restriction fragment length polymorphism (RFLP) analysis, using the 27f/1492r primer set (Supplementary Table S1). PCR was carried out in a 25 µl reaction mixture consisting of 5 µl MangoTaq Buffer (5×, Bionline, Alexandria, Australia), 1.5 mM MgCl₂, 200 µM dNTPs, 10 pmol of each primer, 0.5 µL of resuspended bacterial cells, and 0.5 U MangoTaq DNA polymerase (Bionline). PCR amplification was carried out in an S1000 Thermal Cycler (Bio-Rad, Gladesville, Australia) with PCR programme No. 1 (Suppl. Table S2). The PCR products were digested with *Taq*^α*I* and *Hha**I* restriction enzymes (New England Biolabs, Ipswich, MA, USA), and the digested PCR products were separated on a 2 % (w/v) agarose gel. Sequencing of the 16S rRNA gene fragment was carried out by AGRF (Westmead, NSW, Australia) with 27f primer.

DNA extraction and PCR denaturing gradient gel electrophoresis (DGGE)

DNA was extracted from the rhizosphere (roots and attached soil) using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's protocols. Purified rhizosphere DNA was stored at –20 °C.

Group-specific 16S rRNA DGGE-PCR

Alphaproteobacteria, *Betaproteobacteria*, *Gammaproteobacteria*, *Bacteroidetes*, *Firmicutes*,

Table 1 List of the split-root treatments, combination of the soils filled in the two pots, and the designated rhizosphere sample name

	Split-root treatment	Combination of two pots	Name of rhizosphere sample
(a)	Heterogeneous	+ pyrene or phenanthrene uncontaminated	<i>Het-Pyr</i> or <i>Het-Phe</i> <i>Het-C_{pyr}</i> or <i>Het-C_{phe}</i>
(b)	Homogeneous-PAH (PAH-contaminated control)	+ pyrene or phenanthrene + pyrene or phenanthrene	<i>Hom-Pyr</i> or <i>Hom-Phe</i>
(c)	Homogeneous-control (uncontaminated control)	uncontaminated uncontaminated	<i>Hom-C</i>

Acidobacteria, *Actinobacteria* and *Verrucomicrobia* communities colonizing the split-rhizospheres were analyzed by targeting the respective 16S rRNA genes. The PCR was carried out in a 25 μ L reaction mixture consisting of 5 μ L PCR Reaction Buffer A (5 \times , Kapa Biosystem, Inc., Woburn, MA, USA), 200 μ M dNTPs, 10 pmol of each primer, 0.5 units KAPA 2G Robust DNA polymerase (Kapa Biosystem), and 0.5 μ L of 10-fold diluted rhizosphere DNA (~10 ng) as the template, and the PCR amplification was carried out in an S1000 Thermal Cycler (Bio-Rad). Group-specific amplification of 16S rRNA genes was done with a two-step PCR protocol, using the primer sets listed (Suppl. Tables S1 and S3). The first round PCRs were conducted with the group-specific primer sets, and a 10-fold dilution of the first round PCR product was used as template for the second round (semi-) nested DGGE-PCR. Both PCRs were carried out using PCR programme No. 2 (Suppl. Table S2), with the reaction mixture stated above.

PAH ring-hydroxylating dioxygenase gene DGGE-PCR

The diversity of PAH ring-hydroxylating dioxygenase alpha subunit genes from Gram-positive bacteria (PAH-RHD $_{\alpha}$ -GP) was analyzed with DGGE. PAH-RHD $_{\alpha}$ -GP genes were amplified by PCR from rhizosphere DNA using the PAH-RHD $_{\alpha}$ GP_F/PAH-RHD $_{\alpha}$ GP_R primer set (Suppl. Table S1) (Cébron et al. 2008). A 10-fold dilution of this first round PCR product was then used as the template for the subsequent second round DGGE-PCR with the PAH-RHD $_{\alpha}$ GP_F/GC-PAH-RHD $_{\alpha}$ GP_R primer set. Both PCRs were carried out in a 25 μ L reaction mixture consisting of 5 μ L *MangoTaq* Buffer (5 \times , Bioline), 1.5 mM MgCl₂, 200 μ M dNTPs, 40 pmol of each primer (10 pmol for the second round DGGE-PCR), 2.5 μ g BSA, 2 % DMSO (*v/v*), 5 % acetamide (*w/v*), 0.5 μ L of template, and 0.5 units of *MangoTaq* DNA polymerase (Bioline). PCR

programme No. 3 (Suppl. Table S2) was used (40 cycles amplification for the first round PCR and 30 cycles for the second round DGGE-PCR).

DGGE analysis

Denaturant gradients used for the group specific 16S DGGEs are listed in Supplementary Table S3, while for PAH-RHD $_{\alpha}$ -GP DGGE, 50–70 % denaturant gradient was used. Gels were loaded with the DGGE-PCR products (200 ng/sample) and electrophoresis was conducted at 60 °C for a total of 1008 V or 1700 V hours depending on the DGGE-PCR product size. After completion, the gel was stained with SYBR Gold (Invitrogen), washed briefly with dH₂O, and gel image analysis was carried out with Quantity One V4.6.9 software (Bio-Rad). The presence or absence of bands was scored to give binary matrices for statistical analysis.

Quantitative PCR of PAH-catabolic genes

RNA and DNA extraction, and cDNA synthesis

RNA and DNA were extracted from rhizosphere soil (2 g) with RNA PowerSoil Total RNA Isolation Kit (MO BIO). RNA was extracted according to the manufacturer's protocol, and eluted in 100 μ L of RNase/DNase-free water. DNA was then eluted from the same RNA Capture Column using 4 mL of DNA elution buffer [1 M NaCl, 50 mM MOPS, 15 % isopropanol, pH 7.0]. The eluted DNA was precipitated with isopropanol, redissolved in 10 mM Tris-HCl (pH 8.5) and further treated with Sephadex G200 resin (Pharmacia Fine Chemicals) to remove humic substances (Miller 2001).

RNA was treated with DNase I (New England Biolabs) and re-purified with RNeasy Mini Kit (QIAGEN, Doncaster, Australia) according to the

manufacturer's protocol. Complete removal of DNA was confirmed by absence of 16S rRNA gene amplification with 27f/1492r primers (Suppl. Table S1). cDNA was synthesized by reverse transcription of purified RNA (0.3–4.7 µg) with random hexamer primers (4 µM) and 400 units M-MuLV reverse transcriptase (New England Biolabs), following the manufacturer's instructions. The resulting cDNA was purified with ISOLATE PCR and Gel Kit (Bioline).

Quantitative PCR

Quantitative PCR (qPCR) was used to quantify copy numbers of 16S rRNA, PAH-RHD_α-GP and catechol 2,3-dioxygenase (C23O) genes in DNA and cDNA samples, using 518f/785r primers, PAH-RHD_α-GP_F/PAH-RHD_α-GP_R primers, and C23Of/C23Or primers respectively (Suppl. Table S1). qPCR was performed in a 5 µl reaction mixture containing 2.5 µl KAPA SYBR FAST qPCR Master Mix (2×, Kapa Biosystems), 2 pmol (16S rRNA and C23O) or 4 pmol (PAH-RHD_α-GP) of each primer, and approximately 5 ng of the rhizosphere DNA or cDNA as template. For C23O qPCR, 0.5 µg BSA and 5 % acetamide (*w/v*) were added to each reaction. The reactions were prepared in 384-well PCR plates (Roche Diagnostics) and the amplification was carried out in a LightCycler 480 Real-Time PCR System (Roche) using PCR programme No. 4 (Suppl. Table S2). SYBR[®] Green I fluorescent signals (λ_{exc} 483 nm; λ_{em} 533 nm) were measured at the primer dimer-dissociation step of 10 s at 80 °C. Standards for the qPCRs were purified 16S rRNA and PAH-RHD_α-GP PCR products from *Mycobacterium vanbaalenii* PYR-1 (DSMZ 7251), and C23O PCR product from *Sphingobium yanoikuyae* B1 (DSMZ 6900). The PCR products were purified (ISOLATE PCR and Gel Kit, Bioline) and quantified (NanoDrop 2000C), and the copy number of the standard amplicons was calculated (amplicon size, 16S: 296 bp, PAH-RHD_α-GP: 292 bp, and C23O: 381 bp). Standard curves were created for 10⁸ to 10³ target gene copies/µL, and gene quantification was carried out with the LightCycler[®] 480 software (Roche).

Root exudate collection and analysis

Root exudates of white clover were collected after 30 days of cultivation. Bottom pots of the split-root system were removed and soil adhering to the roots

was carefully washed off with running tap water. Excess moisture was removed with a paper towel, and each half of the split-root system was separately immersed in 50 mL of ultrapure water for 2 h in the light. The collected root exudate solution was then filtered through a 0.22 µm nylon syringe filter (Millipore) and lyophilized.

Root exudate analysis

Methoximated trimethylsilyl (TMS) derivatives were prepared essentially as described previously (Lisec et al. 2006). An internal standard (1 µg ribitol) was added to the lyophilized root exudate sample, which was then derivatised with 40 µl of methoxyamination reagent [20 mg methoxyamine hydrochloride/mL in anhydrous pyridine] and 70 µl of MSTFA (with 1 % trimethylchlorosilane (TMCS)) (Thermo Fisher Scientific). Samples (1 µL) were separated by capillary gas chromatography on an arylene-modified 5 % diphenyl-95 % dimethyl polysiloxane stationary phase (30 m × 0.25 mm ID × 0.25 µm film thickness with a 10 m “guard column”; Rxi-5SilMS, Restek). The column eluent was ionised by electron impact (70 eV) and mass spectra were collected from 70 to 600 amu at 6.67 scans per sec (GCMS-QP2010Plus, Shimadzu). Metabolites were identified by comparing retention indices and mass spectra with an in-house mass spectral/retention index (RI) library for 130 chemical standards plus the Golm Metabolome Database (GMD) (Schauer et al. 2005). Agilent Fiehn and NIST libraries.

Amino acids were quantified as *tert*-butyldimethylsilyl (*t*-BDMS) derivatives (Mawhinney et al. 1986). Internal standard (0.5 µg norleucine) was added to the lyophilized root exudate sample, which was then derivatised with 50 µl of *N*-methyl-*N*-[*tert*-butyldimethylsilyl]trifluoroacetamide. Samples (1 µL) were separated by capillary gas chromatography on the same Rxi-5SilMS column, the column eluent was ionised by electron impact (70 eV) and mass spectra were collected from 100 to 600 amu at 4 scans per second. Amino acids were identified based on retention indices and mass spectra of authentic standards run under the same conditions.

The concentration of 7,4'-dihydroxy flavone (DHF) in root exudates was measured with reversed-phase HPLC. Root exudate solution was lyophilized, re-dissolved in DMSO (400 µL), and made up to 2 mL with 40 % MeOH. DHF was separated on a

5 μm Hypersil BDS C18 column (Shandon HPLC), with an Alltech C18 guard column (Alltech Associates), using a Dionex HPLC system and Chromeleon software ver.6.8 (Dionex). The flow rate was 1 mL/min and analytes were eluted using a multistep gradient of 40–80 % methanol in 1 % acetic acid. DHF was detected fluorometrically (λ_{exc} 360 nm; λ_{em} 415 nm) with an RF2000 fluorescence detector (Dionex), and compared by co-chromatography with standard DHF (INDOFINE Chemical Company).

Statistical analysis

Mean comparison, one-way analysis of variance (ANOVA) and Tukey's test were conducted using IBM SPSS Statistics 19 (IBM Corporation). For microbial community analysis, presence or absence of DGGE bands was converted into binary matrices, and non-metric multidimensional scaling (NMDS) and permutational multivariate analysis of variance (PERMANOVA) (Anderson 2001) were performed with the Jaccard distance using the Palaeontological Statistics (PAST) package ver.3.07 (Hammer et al. 2001) to assess the similarities between the rhizosphere microbial communities.

Results

Heterogeneous PAH contamination alters the pattern of plant nodulation

The rhizosphere microbial diversity of several legume species has been shown to be influenced by the plant's response to PAH contamination (Kawasaki et al. 2012). In order to investigate how this plant-mediated effect controls the function of the rhizosphere community, we studied the effect of PAH contamination on the clover-rhizobium interaction, since this is a critically important functional interaction for nitrogen fixation. No significant difference in nodulation rates were observed between pyrene-contaminated (*Hom-Pyr*) and uncontaminated (*Hom-C*) homogeneous split-root systems (Fig. 1), demonstrating that pyrene contamination did not itself affect nodulation. In the heterogeneous treatment, however, nodulation of the contaminated roots (*Het-Pyr*) was significantly suppressed, with very few nodules formed, and nodulation of the uncontaminated roots (*Het-C_{pyr}*) was significantly enhanced ($p < 0.05$)

(Fig. 1a). When the two halves of each split-root system were combined (*Het-Pyr/Het-C_{pyr}*, *Hom-Pyr* and *Hom-C*), the nodulation rates per total plant mass between the three split-root systems were not significantly different (Fig. 1b).

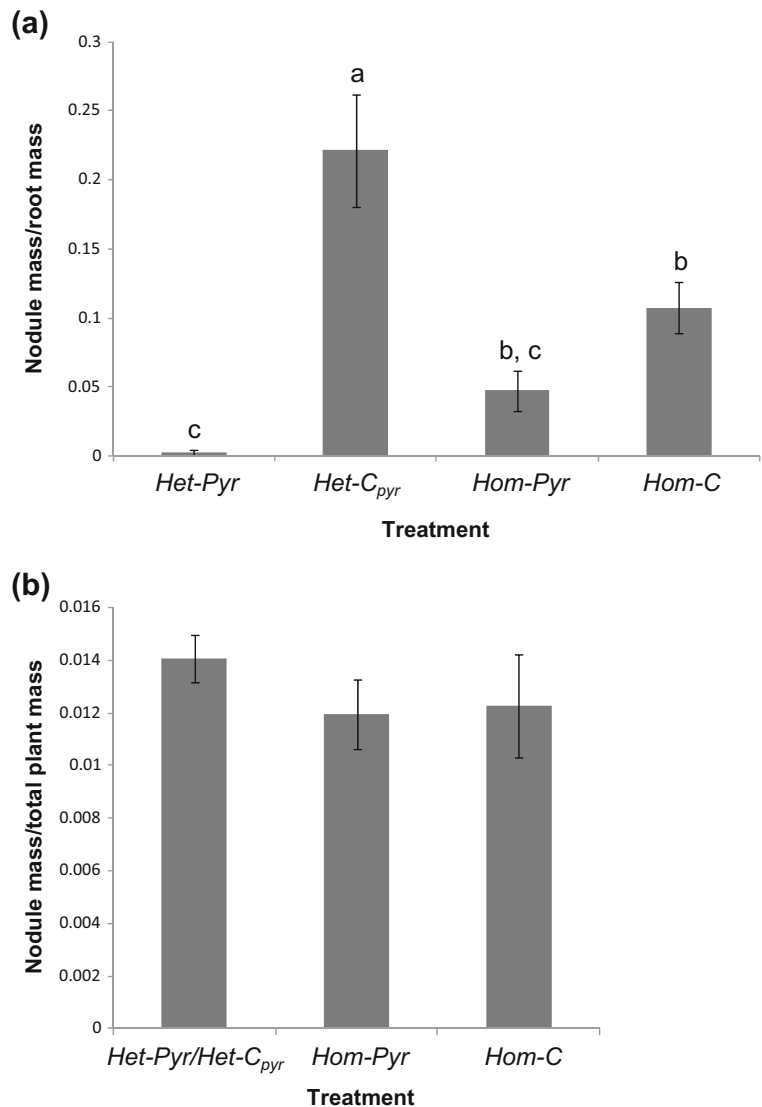
In principle, the observed differences in nodulation rate between the different pyrene treatments could be explained by changed nodule occupancy, with specific rhizobia displaying different sensitivity to PAH contamination. In order to test this, a total of 76 bacterial colonies were isolated from *Het-Pyr*, *Het-C_{pyr}*, *Hom-Pyr* and *Hom-C* nodules, and characterized by 16S-RFLP analysis. The nodules were found to be colonized exclusively by a single bacterial genotype, and DNA sequencing revealed that the isolates all shared >99 % 16S rRNA gene sequence similarity with *Rhizobium leguminosarum*. Susceptibility of the nodule isolates to pyrene was tested in vitro during growth in glucose broth with various concentrations of pyrene. The isolates tested all showed comparable growth rates at a range of pyrene concentrations, even in pyrene-saturated medium, indicating that the rhizobia colonizing the white clover nodules were not susceptible to pyrene contamination (data not shown).

White clover influences specific rhizosphere bacterial groups in response to PAH contamination

The effects of PAH contamination and the plant PAH-stress response on functionally different members of the rhizosphere community were examined in detail by group-specific 16S DGGE of eight bacterial taxa (*Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Bacteroidetes*, *Firmicutes*, *Acidobacteria*, *Actinobacteria* and *Verrucomicrobia*). In homogeneous split-root systems, the PAH-contaminated rhizospheres (*Hom-Pyr* and *Hom-Phe*) and the uncontaminated rhizospheres (*Hom-C*) showed significantly different community structures for almost all the bacterial groups studied ($p < 0.05$) (Suppl. Fig. S1). The only exception here was *Firmicutes* diversity, which did not appear to react to pyrene contamination. The result suggests that PAH exposure is a powerful selective agent for most bacterial taxa in the soil, discriminating for strains that either have enhanced PAH resistance, or are able to degrade PAHs for growth.

Heterogeneous split-root systems revealed that the diversity of all bacterial groups responded strongly to indirect PAH contamination, i.e. mediated by the plant

Fig. 1 Pyrene contamination effects on nodulation of white clover. **a** Ratio of nodule mass to root mass, and **b** ratio of total nodule mass to total plant mass. Data are means \pm standard errors ($n = 5$). Different lower case letters indicate a significant difference ($p < 0.05$) between treatments



PAH-stress response, but that for many groups this differed according to which PAH was applied. For pyrene treatment, *Betaproteobacteria*, *Gammaproteobacteria* and *Acidobacteria* community structures in *Het-C_{pyr}* were significantly different from those in *Hom-C* ($p < 0.05$) (Table 2, and Suppl. Fig. S1b, c and f), indicating that the plant pyrene-stress response affected these groups of bacteria. The same effect was observed with phenanthrene treatment for *Alphaproteobacteria*, *Gammaproteobacteria*, *Bacteroidetes*, *Firmicutes*, *Acidobacteria*, *Actinobacteria* and *Verrucomicrobia* ($p < 0.05$) (Table 2, and Suppl. Fig. S1a, c, d, e, f, g and h). Since the bacterial taxa affected in this experiment had not been directly exposed to the respective PAHs,

this suggested that the plant-response was selecting for particular microbial functions in its rhizosphere as a response to a specific PAH treatment.

Indirect PAH contamination effect influences the diversity of rhizosphere PAH-degraders, but not their activity

White clover plants are placed under considerable stress by the PAH concentrations used in this study with up to 50 % growth inhibition (Kawasaki et al. 2012). We hypothesized that the PAH-degrading community in the rhizosphere may be specifically enriched or stimulated by the plants, as a defense response against PAH-

Table 2 Bacterial groups that are influenced by the plant PAH-stress response in white clover rhizospheres. Group-specific differences in the bacterial community structure between *Het-C_{pyr}* or *Het-C_{phe}* and *Hom-C* were calculated from the 16S DGGE

fingerprints (presence or absence of bands) by PERMANOVA using Jaccard distance. Bacterial groups which were significantly affected ($p < 0.05$) and unaffected ($p > 0.05$) by the indirect PAH-effect are defined

Effect of plant PAH-stress response on *Het-C_{pyr}* and *Het-C_{phe}* bacterial communities

Significantly affected	<i>p</i> values (< 0.05)	Not affected	<i>p</i> values (> 0.05)
<i>Alphaproteobacteria</i> (Phe)	0.024	<i>Alphaproteobacteria</i> (Pyr)	0.096
<i>Betaproteobacteria</i> (Pyr)	0.025	<i>Betaproteobacteria</i> (Phe)	0.060
<i>Gammaproteobacteria</i> (Pyr)	0.012	<i>Bacteroidetes</i> (Pyr)	0.164
<i>Gammaproteobacteria</i> (Phe)	0.013	<i>Firmicutes</i> (Pyr)	0.111
<i>Bacteroidetes</i> (Phe)	0.036	<i>Actinobacteria</i> (Pyr)	0.110
<i>Firmicutes</i> (Phe)	0.023	<i>Verrucomicrobia</i> (Pyr)	0.074
<i>Acidobacteria</i> (Pyr)	0.012		
<i>Acidobacteria</i> (Phe)	0.033		
<i>Actinobacteria</i> (Phe)	0.012		
<i>Verrucomicrobia</i> (Phe)	0.012		

Pyr pyrene treatment in the heterogenous split-root system, *Phe* phenanthrene treatment in the heterogenous split-root system

stress. In order to test this, the diversity and activity of the PAH-degrading community were assessed by targeting two genes involved in PAH degradation those encoding the alpha subunit of the PAH ring-hydroxylating dioxygenase (PAH-RHD $_{\alpha}$), which catalyzes the first PAH-degradation step (Cébron et al. 2008; Habe and Omori 2003), and catechol 2,3-dioxygenase, a general aromatic catabolic enzyme.

Amplification of bacterial PAH dioxygenase genes in the tested rhizospheres using the published primers for Gram-negative and Gram-positive PAH-RHD $_{\alpha}$ genes (Cébron et al. 2008) revealed that PAH-degraders in the white clover rhizospheres were dominated by Gram-positive bacteria, as we could not detect Gram-negative PAH-RHD $_{\alpha}$ genes in this soil (although the Gram-negative genes were detected in other rhizosphere soil samples (data not shown)). DGGE community profiling of the Gram-positive PAH-RHD $_{\alpha}$ (PAH-RHD $_{\alpha}$ -GP) gene diversity in the homogeneous split-root rhizospheres (Fig. 2) showed that PAH-contaminated rhizospheres (*Hom-Pyr* and *Hom-Phe*) clearly differed from the uncontaminated rhizosphere (*Hom-C*) (PERMANOVA *p* values of 0.059 (*Hom-Pyr* and *Hom-C*) and 0.005 (*Hom-Phe* and *Hom-C*)). PAH contamination therefore has a significant influence in changing the diversity of PAH-RHD $_{\alpha}$ -GP gene possessing communities (Fig. 2). For the heterogeneous pyrene treatment, however, the PAH-RHD $_{\alpha}$ -GP community in heterogeneous-control rhizosphere *Het-C_{pyr}* was significantly different from that

of the homogeneous-control rhizosphere *Hom-C* (PERMANOVA *p* value 0.029), and was more similar to those of the pyrene-contaminated *Het-Pyr* and *Hom-Pyr* rhizospheres (Fig. 2a). This indirect effect of pyrene contamination on the PAH-RHD $_{\alpha}$ -GP community suggests that the plant actively shapes the PAH-degrading community in its rhizosphere in response to the pyrene-stress. However, this indirect effect was not observed for phenanthrene contamination, as the PAH-RHD $_{\alpha}$ -GP gene community in *Het-C_{phe}* was not different from *Hom-C* (PERMANOVA *p* value 0.341) (Fig. 2b).

The indirect effect of pyrene contamination on the abundance and activity of the PAH-degrading community in the test rhizospheres was measured by qPCR of the PAH-RHD $_{\alpha}$ -GP, C23O and 16S rRNA genes. Total rhizosphere DNA and RNA (transformed to cDNA) were used to assess abundance and activity of the population respectively. No significant difference in the total bacterial population was observed for the different treatments (Fig. 3c, 16S rRNA gene qPCR). Although the total bacterial activity (16S cDNA) in *Het-Pyr* was significantly lower than in *Hom-C* ($p = 0.046$), no significant difference was seen between *Hom-Pyr* and *Hom-C*, indicating that this effect was not directly due to pyrene contamination (Fig. 3c). In contrast to the 16S rRNA gene results, significant changes in PAH-RHD $_{\alpha}$ -GP gene copy number were observed in both DNA and cDNA samples. Rhizospheres contaminated with pyrene (*Het-Pyr* and *Hom-Pyr*) contained a significantly

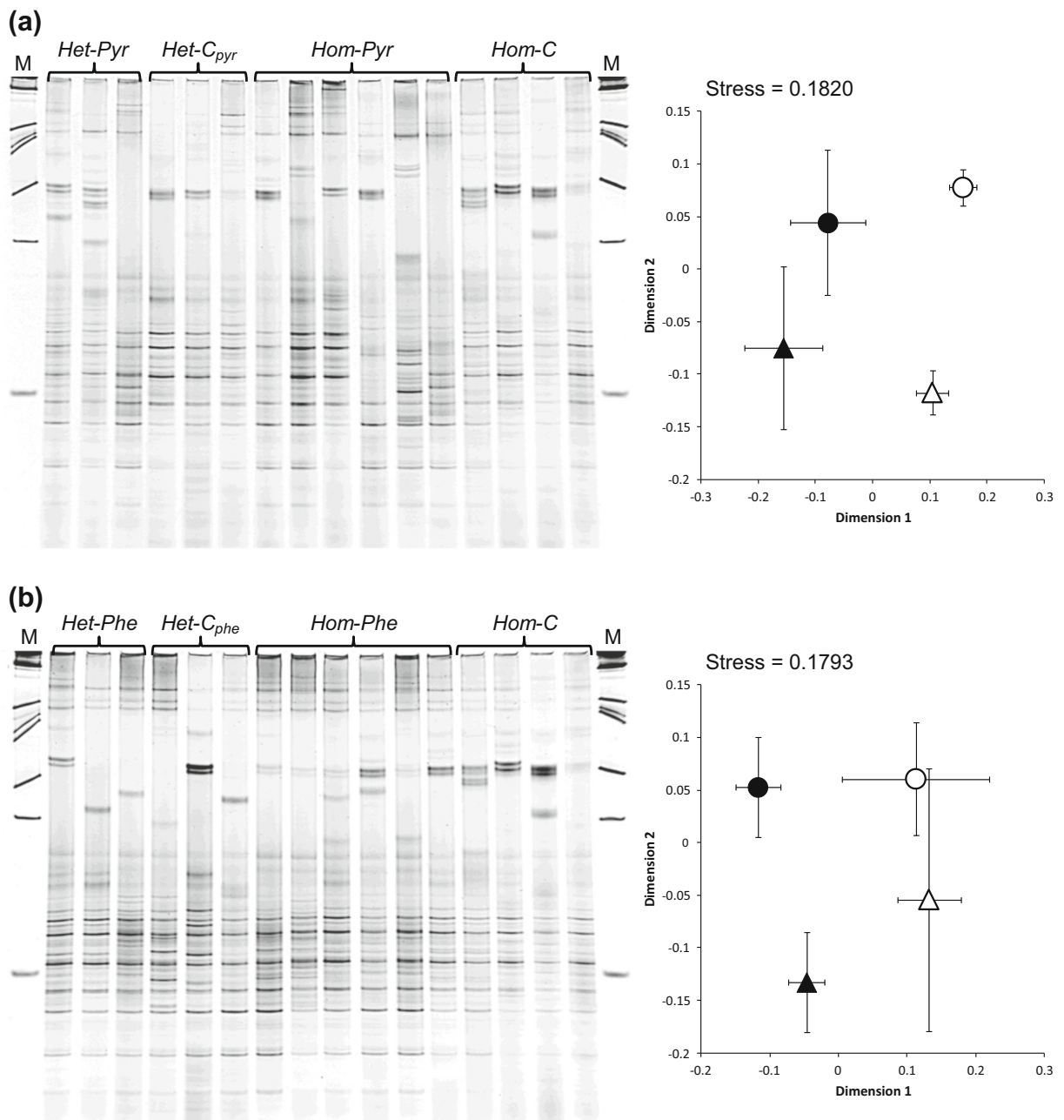


Fig. 2 DGGE profiles and NMDS ordination plots of the Gram-positive bacteria PAH-dioxygenase (PAH-RHD $_{\alpha}$ -GP) gene community in white clover rhizospheres. **a** pyrene or **b** phenanthrene

contamination. M on the DGGE gel represents a marker lane. \blacktriangle : *Het-Pyr* or *Het-Phe*, \triangle : *Het-C_{pyr}* or *Het-C_{phe}*, \bullet : *Hom-Pyr* or *Hom-Phe*, and \circ : *Hom-C*. Data are means \pm standard errors ($n = 3-6$)

higher population (DNA sample) and activity (cDNA sample) of bacteria possessing the PAH-RHD $_{\alpha}$ -GP genes, compared to the uncontaminated rhizospheres (*Het-C_{pyr}* and *Hom-C*) ($p < 0.05$) (Fig. 3a). However, there was no significant difference in PAH-RHD $_{\alpha}$ -GP expression between *Het-C_{pyr}* and *Hom-C* ($p > 0.05$),

indicating that the indirect PAH-effect mediated by the plant does not affect the population size and activity of the PAH-RHD $_{\alpha}$ -GP gene possessing community (Fig. 3a). No significant differences in the C23O gene abundance and expression were observed between the treatments ($p > 0.05$) (Fig. 3b), showing that even direct

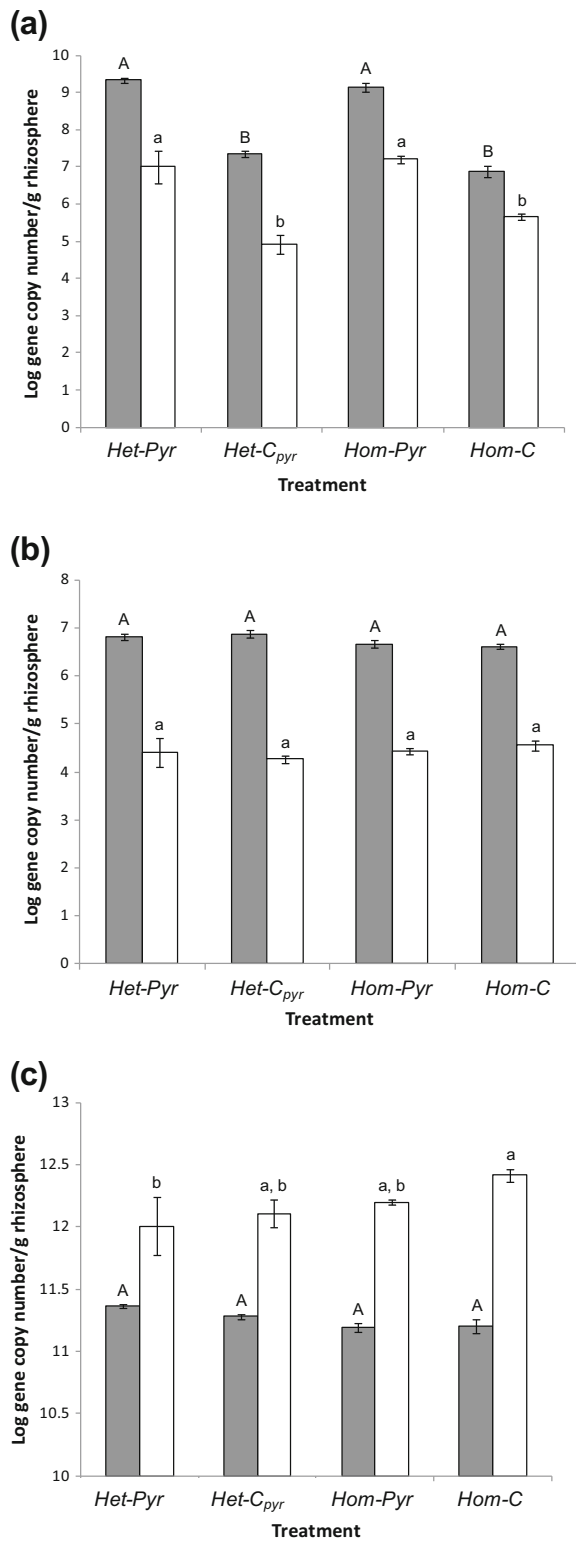


Fig. 3 qPCR quantification of **a** PAH-RHD α -GP, **b** C23O and **c** 16S rRNA genes in both DNA (grey bars) and RNA (cDNA, white bars) samples, in white clover pyrene split-root rhizospheres. Data are means \pm standard errors ($n = 3-6$). Different upper case or lower case letters above the bars indicate a significant difference ($p < 0.05$) between the treatments

Hence, although the plant-mediated indirect pyrene-effect influenced the diversity of pyrene-degraders in the rhizosphere, increases in PAH-RHD α -GP abundance or activity required direct exposure to the contaminant.





Response of white clover root exudates to pyrene treatment

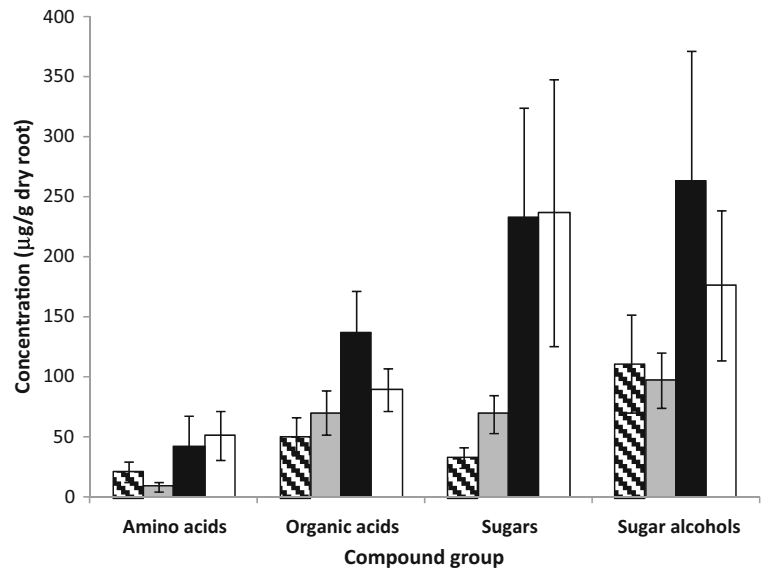
Root exudates have a significant effect in shaping the rhizosphere microbial community structure. Specific exudate compounds released by white clover in response to pyrene-stress may be responsible for the observed rhizosphere diversity response. Therefore, the concentrations of major exudate components (amino acids, organic acids, sugars and sugar alcohols) and of a flavonoid were investigated.

Root exudates were collected from each half of the split-root systems after 30 days of cultivation. A total of 16 amino acids and 25 other metabolites (organic acids, sugars, and sugar alcohols) were detected in the exudates by GC-MS (Fig. 4). The mean total amino acids exuded from the roots within the 2 h collection time were 20.9, 8.7, 42.3 and 51.2 $\mu\text{g/g}$ dry root for *Het-Pyr*, *Het-C_{pyr}*, *Hom-Pyr* and *Hom-C* respectively. For other metabolites, the mean total amounts were 190.1, 232.5, 626.1 and 489.9 $\mu\text{g/g}$ dry root in *Het-Pyr*, *Het-C_{pyr}*, *Hom-Pyr* and *Hom-C* respectively. The measured values ranged from 0.074 $\mu\text{g/g}$ dry root (sorbitol) to 81.4 $\mu\text{g/g}$ dry root (pinitol), but there was no significant difference in the concentration of any of these compounds between treatments ($p > 0.05$) (Fig. 4). Changes in the exudation of these major metabolic compounds are therefore not directly linked with the plant-mediated rhizosphere diversity effects of PAH treatments.

Plant flavonoids are key signalling compounds that are involved in legume nodulation, which was significantly affected by heterogeneous pyrene contamination (Fig. 1a). To test whether these compounds are also involved in indirect signalling of pyrene-stress, concentrations of 7,4'-dihydroxyflavone (DHF, inducer of *R. leguminosarum* bv. *trifolii* nod gene (Djordjevic et al. 1987). in the root exudates were measured. HPLC

pyrene contamination did not significantly enrich the general aromatic-degrading microbial community.

Fig. 4 Concentration of compound groups in white clover root exudates under different split-root treatments. Exudate organic compounds were identified by GC-MS, and classified into four main groups; amino acids, organic acids, sugars and sugar alcohols. : *Het-Pyr*, : *Het-C_{pyr}*, : *Hom-Pyr*, and : *Hom-C*. Data are means \pm standard errors ($n = 3-10$)



analysis did not reveal significant differences in DHF concentration ($p > 0.05$) between exudate samples from different treatments, although root exudates from *Hom-Pyr* contained slightly higher concentrations of DHF compared to the heterogeneously-treated *Het-Pyr* and *Het-C_{pyr}* samples ($p = 0.085$ and 0.058 respectively) (Fig. 5).

Discussion

PAH-degradation in the plant rhizosphere is enhanced by the general enrichment of the microbial population caused by the rhizosphere effect, but it can also be stimulated by specific enrichment of PAH-degrading microbial community (Siciliano and Germida 1998; Siciliano et al. 2003). Legume plants specifically influence the microbial community structure of their rhizosphere in response to PAH-stress (Kawasaki et al. 2012), but the plant's influence on rhizosphere function in response to PAH-stress remains largely unknown. In the present study, community changes in white clover rhizosphere driven by the plant PAH-stress response were examined. We tested whether plants specifically recruit PAH-degraders and stimulate their PAH-catabolic activities in the rhizosphere in response to PAH-stress. The results reported here show clearly that white clover influences the diversity of various bacterial groups in its rhizosphere in response to PAH-stress, including the PAH-degrading community. However, this plant-mediated indirect PAH-effect did not stimulate PAH-

catabolic gene expression, and the microbial population shift was not driven by the root exudation of major plant metabolites.

Many different bacterial taxa are able to degrade PAHs (Fernandez-Luqueno et al. 2011). *Alpha-*, *Beta-*, *Gammaproteobacteria* and *Actinobacteria* include various known PAH-degrading taxa such as sphingomonads, *Burkholderia*, *Pseudomonas*, and *Mycobacterium* respectively (Cunliffe and Kertesz 2006; Khan et al. 2002; Kim et al. 2003; Ma et al. 2006). The community structure of all these groups was significantly affected by the plant-mediated indirect PAH-effect (Table 2 and

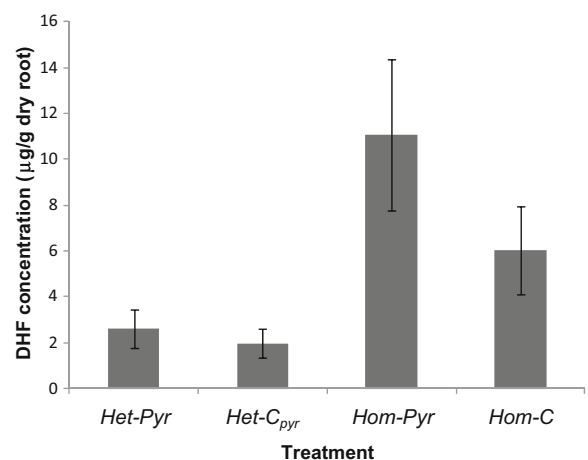


Fig. 5 7,4'-Dihydroxyflavone (DHF) concentration in root exudates collected from white clover pyrene split-root systems. DHF was quantified by reversed-phase HPLC, with fluorometric detection. Data are means \pm standard errors ($n = 5-10$)

Suppl. Fig. S1), suggesting that they play an important role in the plant's PAH-stress response. Interestingly, *Acidobacteria* and *Verrucomicrobia* diversity was also affected by the plant PAH-response (Table 2). Increased *Verrucomicrobia* population was previously observed in pyrene-contaminated white clover rhizospheres (Kawasaki et al. 2012). although PAH-degradation by *Acidobacteria* and *Verrucomicrobia* has never been reported. However, the rhizosphere population of these bacterial groups may respond differently to PAH depending on the soil type and plant species. For instance, when a birch rhizosphere was contaminated with PAHs, a community which was predominantly composed of *Acidobacteria* and *Verrucomicrobia* shifted to become *Alphaproteobacteria* and *Betaproteobacteria*-dominated (Yrjälä et al. 2010).

Direct and indirect plant-mediated effects of PAH contamination on the functional changes in the rhizosphere PAH-degrading community and activity were investigated in this work by targeting genes encoding the alpha subunit of PAH ring-hydroxylating dioxygenase (PAH-RHD $_{\alpha}$). In the soil tested, amplification with published PCR probes for this gene showed that the PAH-degrading community was dominated by Gram-positive organisms. Although this may be soil-dependent, it is interesting that in another legume, alfalfa, the density of Gram-positive bacteria bearing the PAH-RHD $_{\alpha}$ gene also increased by about 10-fold during plant growth (Cébron et al. 2009). This correlates with the enrichment of *Bacillus*, *Paenibacillus* and *Mycobacterium* observed previously in pyrene-contaminated white clover rhizospheres (Kawasaki et al. 2012). and suggests that these may be key species that are directly enriched in the presence of pyrene, and responsible for PAH-degradation in this system. However, one of the most important findings of this study is that the PAH-RHD $_{\alpha}$ -GP community in the pyrene-contaminated heterogeneous split-root system responds not only to direct PAH contamination, but also to the indirect PAH-effect mediated by the plant. The PAH-RHD $_{\alpha}$ -GP community structure in *Het-C_{pyr}* was significantly different from *Hom-C*, but it was more similar to *Hom-Pyr* and *Het-Pyr* (Fig. 2a). However, the abundance and expression of PAH-RHD $_{\alpha}$ -GP and C23O genes was not stimulated by the indirect PAH-effect, (Fig. 3a and b). This indicates that white clover can specifically alter the root colonizing PAH-degrading population in response to pyrene-stress, but at the time of sampling (after 30 days cultivation), the plant does not directly stimulate expression of PAH-

degrading genes in the rhizosphere to increase PAH-degradation.

The abundance and expression of the PAH-catabolic genes were only measured at one time point, at the end of the plant cultivation, and it is possible that catabolic gene expression was stimulated at earlier stages of plant growth. Abundance and expression of PAH-RHD $_{\alpha}$ genes is highly dependent on the sampling time and the type of PAH. Time course measurements of PAH-RHD $_{\alpha}$ gene abundance in PAH-contaminated soils revealed that addition of benzo(a)pyrene resulted in a 3-fold increase of the Gram-negative PAH-RHD $_{\alpha}$ gene abundance from day 2 to day 20, while contamination with phenanthrene or fluoranthene did not affect the gene abundance level over the monitoring period (Sawulski et al. 2014). Expression of ring-hydroxylating dioxygenase (RHD) genes was monitored at the early stage of petroleum contamination in a microcosm, and the gene expression reached the peak at 12 h after addition of petroleum, and no expression was detected after 24 h (Paisse et al. 2012). Thus, time course monitoring may be able to elucidate the stimulation of the PAH-catabolic gene abundance and expression by the indirect PAH-effect in the white clover rhizospheres.

Plant interactions with rhizosphere microbes are largely controlled by root exudate rates, either through the production of specific signal molecules, as for nodulation, or by compositional changes in bulk exudate selecting for specific microbial functional groups. Plants change the root exudation rate and composition to protect themselves from various biotic and abiotic negative stresses (Badri and Vivanco 2009). including PAH-stress (Muratova et al. 2009a; Muratova et al. 2009b; Xie et al. 2009). Addition of plant root exudates to soils has been shown to enhance both the number of PAH-degrading bacteria (Miya and Firestone 2001; Siciliano et al. 2003; Yoshitomi and Shann 2001). and the diversity of PAH-degraders (Cébron et al. 2011). For example, addition of ryegrass root exudates to an aged PAH-contaminated soil caused a shift in phenanthrene-degraders from *Pseudoxanthomonas* spp. and *Microbacterium* spp. to a population dominated by *Arthrobacter* spp. and *Pseudomonas stutzeri* (Cébron et al. 2011). The plant-mediated shifts in the bacterial population of the white clover rhizosphere that were observed after PAH-stress in this work might also be driven by changes in the plant root exudation. However, no significant differences were observed between the exudates collected from control plants and those from

PAH-exposed plants, for any compounds in the main metabolite classes (Fig. 4). These metabolites are probably not the key signalling compounds that plants release in response to PAH-stress, since alfalfa and wild rye also did not show significant changes in their root exudation pattern for amino acids, organic acids and phenolics when exposed to PAHs (phenanthrene and pyrene) (Phillips et al. 2012).

Flavonoids are further components of root exudates which are actively secreted from plant roots. They have a key signalling role to mediate plant nodulation as the inducers of rhizobium *nod* genes, (Hassan and Mathesius 2012), and are also involved in the plant defence response against various parasites and pathogens. Their concentration in root exudates increases in response to various microbial signals and abiotic stresses (e.g. water, nitrogen and temperature) (Weston and Mathesius 2013). When the heterogeneous split-root system was stressed with pyrene, white clover responded by inhibiting nodulation on contaminated roots (*Het-Pyr*) but significantly enhancing nodule development on the control uncontaminated roots (*Het-C_{pyr}*) (Fig. 1a). Enhanced or suppressed nodulation on one side of a split-root system has been reported before. For instance, a time delay between inoculation of the two sides of split-root soybeans with *Rhizobium* led to significant inhibition of nodulation on the delayed side (Kosslak and Bohlool 1984), and a similar result was obtained with *Lotus japonicus* and *Mesorhizobium loti* (Suzuki et al. 2008). This autoregulation of nodulation is regulated through shoot-root signalling by peptide hormones, and responds strongly to nitrate concentrations in the plant, and to ethylene as a stress response molecule (Ferguson et al. 2010; Suzuki et al. 2008). The suppression of nodulation seen in the *Het-Pyr* rhizosphere may be caused by a delay in the *Rhizobium* infection relative to the *Het-C_{pyr}* rhizosphere, or by synthesis of ethylene as a stress response to pyrene contamination. Nevertheless, it is interesting that this is apparent on a local level, and not systemically, since the total number of nodules produced by the plant was unchanged (Fig. 1b). *Rhizobium leguminosarum* bv. *trifolii* is relatively resistant to PAH contamination in soil (our data, and (Johnson et al. 2004)). This suggests that rather than inhibiting the microbes, PAH may interfere with plant-microbe signalling, either reducing the synthesis of root-secreted flavonoids or interfering with reception of the flavonoid signal by the rhizobia (Wetzel and Werner 1995). Since flavonoid (DHF)

concentrations in the exudates were not significantly different between treatments (Fig. 5), the latter appears to be more likely. Other plant products that have been shown to be involved in stimulating bacterial pyrene-degradation include linoleic acid, which stimulates *Mycobacterium vanbaalenii* PYR-1 (Yi and Crowley 2007), and secondary metabolites that are structurally similar to xenobiotics, including PAH-analogues (Perry et al. 2007; Singer et al. 2003). It is possible that plants release these at low concentrations in response to PAH-stress, in order to attract and stimulate microbes which can degrade the secondary metabolites and cometabolize PAHs, but these were below the detection limit of the current study.

Further investigation on the mechanism of plant-microbes signalling in the PAH-contaminated rhizosphere needs to address these minor components of exudates, in the search for the signalling molecules concerned.

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References

- Anderson MJ (2001) A new method for non-parametric multivariate analysis of variance. *Austral Ecol* 26:32–46. doi:10.1111/j.1442-9993.2001.01070.pp.x
- Anderson TA, Guthrie EA, Walton BT (1993) Bioremediation in the rhizosphere. *Environ Sci Technol* 27:2630–2636. doi:10.1021/es00049a001
- Badri DV, Vivanco JM (2009) Regulation and function of root exudates. *Plant Cell Environ* 32:666–681. doi:10.1111/j.1365-3040.2009.01926.x
- Berg G, Smalla K (2009) Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. *FEMS Microbiol Ecol* 68:1–13. doi:10.1111/j.1574-6941.2009.00654.x
- Brimecombe MJ, De Leij FAAM, Lynch JM (2007) Rhizodeposition and microbial populations. In: Pinton R, Varanini Z, Nannipieri P (eds) *The rhizosphere*, 2nd edn. CRC Press, Boca Raton
- Cébron A, Norini MP, Beguiristain T, Leyval C (2008) Real-time PCR quantification of PAH-ring hydroxylating dioxygenase (PAH-RHD alpha) genes from Gram positive and Gram negative bacteria in soil and sediment samples. *J Microbiol Methods* 73:148–159. doi:10.1016/j.mimet.2008.01.009
- Cébron A, Beguiristain T, Faure P, Norini MP, Masfarauud JF, Leyval C (2009) Influence of vegetation on the *in situ* bacterial community and polycyclic aromatic hydrocarbon (PAH)

- degraders in aged PAH-contaminated or thermal-desorption-treated soil. *Appl Environ Microbiol* 75:6322–6330. doi:10.1128/aem.02862-08
- Cébron A, Louvel B, Faure P, France-Lanord C, Chen Y, Murrell JC, Leyval C (2011) Root exudates modify bacterial diversity of phenanthrene degraders in PAH-polluted soil but not phenanthrene degradation rates. *Environ Microbiol* 13:722–736. doi:10.1111/j.1462-2920.2010.02376.x
- Cerniglia CE (1992) Biodegradation of polycyclic aromatic hydrocarbons. *Biodegradation* 3:351–368
- Corgié SC, Joner EJ, Leyval C (2003) Rhizospheric degradation of phenanthrene is a function of proximity to roots. *Plant Soil* 257:143–150. doi:10.1023/A:1026278424871
- Cunliffe M, Kertesz MA (2006) Autecological properties of soil sphingomonads involved in the degradation of polycyclic aromatic hydrocarbons. *Appl Microbiol Biotechnol* 72:1083–1089. doi:10.1007/s00253-006-0374-x
- Djordjevic MA, Redmond JW, Batley M, Rolfe BG (1987) Clovers secrete specific phenolic compounds which either stimulate or repress *nod* gene expression in *rhizobium trifolii*. *Embo j* 6:1173–1179
- Ferguson BJ, Indrasumunar A, Hayashi S, Lin MH, Lin YH, Reid DE, Gresshoff PM (2010) Molecular analysis of legume nodule development and autoregulation. *J Integr Plant Biol* 52:61–76. doi:10.1111/j.1744-7909.2010.00899.x
- Fernandez-Luqueno F, Valenzuela-Encinas C, Marsch R, Martinez-Suarez C, Vazquez-Nunez E, Dendooven L (2011) Microbial communities to mitigate contamination of PAHs in soil—possibilities and challenges: a review. *Environ Sci Pollut Res* 18:12–30. doi:10.1007/s11356-010-0371-6
- Habe H, Omori T (2003) Genetics of polycyclic aromatic hydrocarbon metabolism in diverse aerobic bacteria. *Biosci Biotechnol Biochem* 67:225–243
- Hammer Ø, Harper DAT, Ryan PD (2001) PAST: paleontological statistics software package for education and data analysis. *Palaeontol Electron* 4:1–9
- Hassan S, Mathesius U (2012) The role of flavonoids in root-rhizosphere signalling: opportunities and challenges for improving plant-microbe interactions. *J Exp Bot* 63:3429–3444. doi:10.1093/jxb/err430
- Jha P, Panwar J, Jha PN (2015) Secondary plant metabolites and root exudates: guiding tools for polychlorinated biphenyl biodegradation. *Int J Environ Sci Technol* 12:789–802. doi:10.1007/s13762-014-0515-1
- Johnson DL, Maguire KL, Anderson DR, McGrath SP (2004) Enhanced dissipation of chrysene in planted soil: the impact of a rhizobial inoculum. *Soil Biol Biochem* 36:33–38. doi:10.1016/j.soilbio.2003.07.004
- Johnson DL, Anderson DR, McGrath SP (2005) Soil microbial response during the phytoremediation of a PAH contaminated soil. *Soil Biol Biochem* 37:2334–2336. doi:10.1016/j.soilbio.2005.04.001
- Kawasaki A, Watson ER, Kertesz MA (2012) Indirect effects of polycyclic aromatic hydrocarbon contamination on microbial communities in legume and grass rhizospheres. *Plant Soil* 358:169–182. doi:10.1007/s11104-011-1089-z
- Khan AA, Kim SJ, Paine DD, Cerniglia CE (2002) Classification of a polycyclic aromatic hydrocarbon-metabolizing bacterium, *mycobacterium* sp. Strain PYR-1, as *mycobacterium vanbaalenii* sp. nov. *Int J Syst Evol Microbiol* 52:1997–2002. doi:10.1099/ijs.0.02163-0
- Kim TJ, Lee EY, Kim YJ, Cho KS, Ryu HW (2003) Degradation of polyaromatic hydrocarbons by *Burkholderia cepacia* 2A-12. *World J Microbiol Biotechnol* 19:411–417. doi:10.1023/a:1023998719787
- Kosslak RM, Bohlool BB (1984) Suppression of nodule development of one side of a split-root system of soybeans caused by prior inoculation of the other side. *Plant Physiol* 75:125–130. doi:10.1104/pp.75.1.125
- Kuiper I, Lagendijk EL, Bloemberg GV, Lugtenberg BJJ (2004) Rhizoremediation: a beneficial plant-microbe interaction. *Mol Plant-Microbe Interact* 17:6–15. doi:10.1094/MPMI.2004.17.1.6
- Lamb EG, Kennedy N, Siciliano SD (2011) Effects of plant species richness and evenness on soil microbial community diversity and function. *Plant Soil* 338:483–495. doi:10.1007/s11104-010-0560-6
- Lisec J, Schauer N, Kopka J, Willmitzer L, Fernie AR (2006) Gas chromatography mass spectrometry-based metabolite profiling in plants. *Nat Protoc* 1:387–396. doi:10.1038/nprot.2006.59
- Ma YF, Wang L, Shao ZZ (2006) *Pseudomonas*, the dominant polycyclic aromatic hydrocarbon-degrading bacteria isolated from antarctic soils and the role of large plasmids in horizontal gene transfer. *Environ Microbiol* 8:455–465. doi:10.1111/j.1462-2920.2005.00911.x
- Marschner P, Crowley D, Yang CH (2004) Development of specific rhizosphere bacterial communities in relation to plant species, nutrition and soil type. *Plant Soil* 261:199–208. doi:10.1023/B:PLSO.0000035569.80747.c5
- Mawhinney TP, Robinett RSR, Atalay A, Madson MA (1986) Analysis of amino acids as their *tert*-butyldimethylsilyl derivatives by gas-liquid chromatography and mass spectrometry. *J Chromatogr* 358:231–242. doi:10.1016/S0021-9673(01)90333-4
- Millard P, Singh BK (2010) Does grassland vegetation drive soil microbial diversity? *Nutr Cycl Agroecosyst* 88:147–158. doi:10.1007/s10705-009-9314-3
- Miller DN (2001) Evaluation of gel filtration resins for the removal of PCR-inhibitory substances from soils and sediments. *J Microbiol Methods* 44:49–58. doi:10.1016/S0167-7012(00)00228-1
- Miya RK, Firestone MK (2001) Enhanced phenanthrene biodegradation in soil by slender oat root exudates and root debris. *J Environ Qual* 30:1911–1918
- Muratova A, Golubev S, Wittenmayer L, Dmitrieva T, Bondarenkova A, Hirche F, Merbach W, Turkovskaya O (2009a) Effect of the polycyclic aromatic hydrocarbon phenanthrene on root exudation of *Sorghum bicolor* (L.) moench. *Environ Exp Bot* 66:514–521. doi:10.1016/j.envexpbot.2009.03.001
- Muratova A, Pozdnyakova N, Golubev S, Wittenmayer L, Makarov O, Merbach W, Turkovskaya O (2009b) Oxidoreductase activity of sorghum root exudates in a phenanthrene-contaminated environment. *Chemosphere* 74:1031–1036. doi:10.1016/j.chemosphere.2008.11.011
- Paisse S, Goni-Urriza M, Stadler T, Budzinski H, Duran R (2012) Ring-hydroxylating dioxygenase (RHD) expression in a microbial community during the early response to oil pollution. *FEMS Microbiol Ecol* 80:77–86. doi:10.1111/j.1574-6941.2011.01270.x

- Perry LG, Alford ER, Horiuchi J, Paschke MW, Vivanco JM (2007) Chemical signals in the rhizosphere: root-root and root-microbe communication. In: Pinton R, Varanini Z, Nannipieri P (eds) *The rhizosphere*, 2nd edn. CRC Press, Boca Raton
- Phillips LA, Greer CW, Farrell RE, Germida JJ (2012) Plant root exudates impact the hydrocarbon degradation potential of a weathered-hydrocarbon contaminated soil. *Appl Soil Ecol* 52:56–64. doi:10.1016/j.apsoil.2011.10.009
- Pilon-Smits E (2005) Phytoremediation. *Annu Rev Plant Biol* 56: 15–39. doi:10.1146/annurev.arplant.56.032604.144214
- Pritchina O, Ely C, Smets BF (2011) Effects of PAH-contaminated soil on rhizosphere microbial communities. *Water Air Soil Pollut* 222:17–25. doi:10.1007/s11270-011-0800-2
- Sawulski P, Clipson N, Doyle E (2014) Effects of polycyclic aromatic hydrocarbons on microbial community structure and PAH ring hydroxylating dioxygenase gene abundance in soil. *Biodegradation* 25:835–847. doi:10.1007/s10532-014-9703-4
- Schauer N, Steinhauser D, Strelkov S, Schomburg D, Allison G, Moritz T, Lundgren K, Roessner-Tunali U, Forbes MG, Willmitzer L, Fernie AR, Kopka J (2005) GC-MS libraries for the rapid identification of metabolites in complex biological samples. *FEBS Lett* 579:1332–1337. doi:10.1016/j.febslet.2005.01.029
- Siciliano SD, Germida JJ (1998) Mechanisms of phytoremediation: biochemical and ecological interactions between plants and bacteria. *Environ Rev* 6:65–79. doi:10.1139/er-6-1-65
- Siciliano SD, Fortin N, Mihoc A, Wisse G, Labelle S, Beaumier D, Ouellette D, Roy R, Whyte LG, Banks MK, Schwab P, Lee K, Greer CW (2001) Selection of specific endophytic bacterial genotypes by plants in response to soil contamination. *Appl Environ Microbiol* 67:2469–2475. doi:10.1128/AEM.67.6.2469-2475.2001
- Siciliano SD, Germida JJ, Banks K, Greer CW (2003) Changes in microbial community composition and function during a polyaromatic hydrocarbon phytoremediation field trial. *Appl Environ Microbiol* 69:483–489. doi:10.1128/AEM.69.1.483-489.2003
- Singer AC, Crowley DE, Thompson IP (2003) Secondary plant metabolites in phytoremediation and biotransformation. *Trends Biotechnol* 21:123–130. doi:10.1016/s0167-7799(02)00041-0
- Singh BK, Munro S, Potts JM, Millard P (2007) Influence of grass species and soil type on rhizosphere microbial community structure in grassland soils. *Appl Soil Ecol* 36:147–155. doi:10.1016/j.apsoil.2007.01.004
- Sudini H, Liles MR, Arias CR, Bowen KL, Huettel RN (2011) Exploring soil bacterial communities in different peanut-cropping sequences using multiple molecular approaches. *Phytopathology* 101:819–827. doi:10.1094/phyto-11-10-0310
- Suzuki A, Hara H, Kinoue T, Abe M, Uchiumi T, Kucho KI, Higashi S, Hirsch AM, Arima S (2008) Split-root study of autoregulation of nodulation in the model legume *lotus japonicus*. *J Plant Res* 121:245–249. doi:10.1007/s10265-007-0145-5
- Walton BT, Hoylman AM, Perez MM, Anderson TA, Johnson TR, Guthrie EA, Christman RF (1994) Rhizosphere microbial communities as a plant defense against toxic-substances in soils. In: Anderson TA, Coats JR (eds) *Bioremediation through rhizosphere technology*. American Chemical Society, Washington, D.C.
- Weston LA, Mathesius U (2013) Flavonoids: their structure, biosynthesis and role in the rhizosphere, including allelopathy. *J Chem Ecol* 39:283–297. doi:10.1007/s10886-013-0248-5
- Wetzel A, Werner D (1995) Ecotoxicological evaluation of contaminated soil using the legume root-nodule symbiosis as effect parameter. *Environ Toxicol Water Qual* 10:127–133. doi:10.1002/tox.2530100207
- Wilson SC, Jones KC (1993) Bioremediation of soil contaminated with polynuclear aromatic-hydrocarbons (PAHs) - a review. *Environ Pollut* 81:229–249
- Xie MJ, Yan CL, Ye J, Wei LL (2009) Impact of phenanthrene on organic acids secretion and accumulation by perennial ryegrass, *lolium perenne* L., root. *Bull Environ Contam Toxicol* 83:75–80. doi:10.1007/s00128-009-9775-8
- Xuan DT, Guong VT, Rosling A, Alstrom S, Chai BL, Hogberg N (2012) Different crop rotation systems as drivers of change in soil bacterial community structure and yield of rice, *Oryza sativa*. *Biol Fertil Soils* 48:217–225. doi:10.1007/s00374-011-0618-5
- Yi H, Crowley DE (2007) Biostimulation of PAH degradation with plants containing high concentrations of linoleic acid. *Environ Sci Technol* 41:4382–4388. doi:10.1021/es062397y
- Yoshitomi KJ, Shann JR (2001) Corn (*Zea mays* L.) root exudates and their impact on C-14-pyrene mineralization. *Soil Biol Biochem* 33:1769–1776. doi:10.1016/S0038-0717(01)00102-X
- Yrjälä K, Keskinen AK, Åkerman ML, Fortelius C, Sipilä TP (2010) The rhizosphere and PAH amendment mediate impacts on functional and structural bacterial diversity in sandy peat soil. *Environ Pollut* 158:1680–1688. doi:10.1016/j.envpol.2009.11.026