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RHIZOSPHERE BACTERIA COMMUNITY AND PETROL HYDROCARBON (PHC) BIODEGRADATION IN SOIL PLANTED TO FIELD CROPS

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A greenhouse pot experiment was conducted for investigating the capability of a grass (annual ryegrass), a legume (summer vetch), and a crucifer (white mustard) to grow in a soil from a former coal gasification site and promote the biodegradation of petrol hydrocarbons (PHCs). Soil concentrations of 1517 mg kg^{-1} of total petrol hydrocarbons (TPHs), including 71.4 mg kg^{-1} of total US EPA priority polycyclic aromatic hydrocarbons (TPAHs) have caused a significant (P<0.05) reduction in shoot and root yields by more than 50%. Abundance and bacterial community composition in soil and rhizosphere were distinctly altered by the PHC contaminants and also depended on crop species and age. After 95 days, 68.7% of initial TPH amounts and 59% of the TPAHs had disappeared from unplanted soil. The removal of PHCs was fostered in soil planted to mustard and vetch reaching final TPH concentrations that were 15.6% and 12%, respectively, lower than in unplanted soil. Mustard and vetch elicited the greatest degradative root activities and sustained particularly great populations of rhizosphere bacteria that are known hydrocarbon degraders. None of the crops aided the biodegradation of TPAHs in soil.

K e y w o r d s: bacteria; bioavailability; biodegradation; crops; PAHs; petrol hydrocarbons; phytoremediation; rhizosphere.

Introduction

Bioremediation often is used to clean up hazardous waste sites that are contaminated with petrol hydrocarbons, but current *in situ* techniques are often ineffective for the removal of the more recalcitrant, toxic, mutagenic, and carcinogenic PHC constituents such as PAHs [57; 5]. Accelerating the biodegradation of petrol hydrocarbons in general and of PAHs in particular is thus a major challenge to improving the performance and acceptance of cost-saving soil bioremediation techniques.

Plants are suggested to enhance the degradation of organopollutants because of greater numbers and activities of microbes in the rhizosphere [10; 42]. A plant-aided biodegradation was evident for pesticides [57], petrol hydrocarbons [56], PAHs [4; 29] and many other organic compounds [32]. Yet, there is also a number of research studies demonstrating neutral [14] or inhibitory [51] plant effects with many data indicating that plant species effect the fate of particular chemicals differently [7]. A more systematic approach to selecting plant species for phytoremediation purposes is thus essential but has rarely been attempted [11; 56]. Especially differences in microbial community features and contaminant degradation between rhizosphere soils of various plant species or types are not well documented in the literature [7].

The objective of this study was to investigate three field crops from different botanical groups for their rhizosphere bacterial communities and capabilities to grow in a contaminated soil from a former coal gasification site and foster the biodegradation of petrol hydrocarbons, including PAHs. Field crops are of particular interest for phytoremediation purposes because they are bred for fast growth and high yields.

Materials and methods

Soils and treatments

Loamy sand (2.1% organic carbon, pH 7.6) from Berlin and soil contaminated with petrol hydrocarbons (46.3 g TPHs kg^{-1} dry soil), including PAHs (2129.8 mg TPAHs kg^{-1} dry soil) from a former coal gasification plant site in Gröditz, Saxony, were air dried and passed through a 6-mm sieve to remove large rocks and debris. The loamy sand (100 kg) and fertilizer (0.42 kg) (6% NO_3-N, 7% NH_4-N, 13% P_2O_5, 21% K_2O) were placed in a cement mixer and blended for 10 min. The contaminated soil (3350 g) was added to the cement mixer and diluted thoroughly with the fertilized loamy sand for 20 min. The procedure was aimed to have contaminated and control soil of similar physical properties and to

decrease the toxicity of polluted soil. The contaminated loamy sand mixture (2.4% organic carbon, pH 7.7) was stored for equilibration (aging) for 30 days in the dark at 15°C and final concentrations were reached of 1517 mg TPHs kg^{-1} dry soil, including 71.4 mg TPAHs kg^{-1} dry soil.

Portions of 1000 g of the contaminated or uncontaminated loamy sand were placed in black plastic pots (13 cm diam.), and seeds (Saaten-Union GmbH, Hannover, Germany) of summer vetch (*Vicia sativa* L.), white mustard (*Sinapsis alba* L.), and annual ryegrass (*Lolium multiflorum* Lam.) were sown into the soil (15 seeds per pot). Seedlings were later thinned to give five plants per pot. Tap water was added to the soils to bring the moisture level to approximately 80% of the water-holding capacity. The plants were grown in a greenhouse maintained at 15°C during a 16-h day and at 10°C during an 8-h night. The light intensity was according to sunlight conditions of early summer and additional light was provided as needed by sodium vapor lamps (Osram Violox T 400) at an intensity of 10,000 cd m^{-2}. The soils were watered daily and fertilized every two weeks with 10 ml of a 0.3-% solution of Poly Crescal (AGLUKON GmbH, Düsseldorf, Germany) per 100 g of soil. The 12 replicate pots of each treatment were completely randomized in the greenhouse.

Plant analysis

After 95 days of plant growth, from six pots of each treatment the shoots were excised from the roots. All soil was removed from the pots and air dried before the roots that had completely permeated the entire soil could be separated from the planted soil by passing the soil through a 6-mm sieve and picking the remaining roots by hand. The roots and shoots were washed in tap water, dried for 24 h at 105°C, and weighed.

Soil analysis

For extraction of TPHs, air-dried soil (10 g) from each sampled pot was homogenized with 30 g of anhydrous Na_2SO_4 and subjected to extraction using 1 h sonication with 110 ml of 1,1,2-trichlorotrifluoroethane (IR spectroscopy grade, Merck AG, Darmstadt, Germany). The hydrocarbon extracts were cleaned on neutral aluminum oxide powder (50-200 µm, pH 7.0-7.8) and analyzed by an infrared analyzer (Horiba OMCA-200) as TPH-IR according to DIN method 38409-18 (DIN, 1981).

For PAH extraction, air-dried soil (10 g) from each sampled pot was mixed with 20 g of anhydrous Na_2SO_4 and subjected to extraction using 2 h sonication with 100 ml of tetrahydrofuran (Merck AG, Darmstadt, Germany). The PAH extracts were analyzed with HPLC using UV detection based on U.S. EPA Method 610 (US EPA, 1998). Extraction and analysis of TPHs and PAHs were conducted by an accredited commercial service laboratory (Chemisches Labor Dr. Betz GmbH, Berlin, Germany).

Bacterial enumeration and identification

After 15 days and when crops began to bloom (mustard, 57 d; vetch, 61 d) or after 60 days (ryegrass and soil), plants were carefully pulled from six pots of each treatment and mildly shaken to remove soil loosely adhering to the roots. The roots with the remaining adherent soil were separated from the shoots and portions of 1 g (15-day samples) or 10 g (wet wt.) were transferred either to 50-ml glass tubes filled with 9 ml or into 250-ml Erlmeyer flasks containing 90 ml of sterile saline (0.9%). Tubes and flasks were closed with an autoclaved cotton plug, and shaken for 20 min at 200 rev min^{-1} on an orbital shaker (B. Braun Biotech International GmbH, Melsungen, Germany). After allowing the soil slurry to settle for 20 min, 1 ml of the supernatant was serially diluted in 50-ml glass tubes containing 9 ml of sterile saline (0.9%), and aliquots (0.1 ml) of the proper dilution (producing 30 to 300 colonies per plate) were spread on glycerin-peptone agar plates [17] in duplicate. The unplanted soil was processed the same way. The plates were incubated for 5 to 7 days at 25°C prior conducting discriminatory colony counting for determining abundance and composition of the bacterial community. In more detail, all colonies of a plate were grouped and counted according to their morphological appearance [44; 52] and one representative of each colony morphotype (cmt) was isolated and purified. The isolates were taxonomically identified by gas-chromatographic analysis of their whole-cell fatty acid methyl esters (FAME) with the Hewlett-Packard HP5898A Microbial Identification System (MIS®) using versions 3.80 and 4.01 of the Aerobic Library (TSBA 40) (MIDI, Inc., Newark, DE, USA) and according to the procedure as specified by the manufacturer (Hewlett-Packard, Avondale, Pennsylvania, USA). Microbes that vanished during cultivation were summarized as "Deserter" and those that failed FAME identification have been designated by the system as "no match". Organisms not identifiable by FAME analysis were coarsely grouped on the basis of biochemical or colony morphology features. Bacteria were discriminated by Gram reaction using KOH (3%) [45]. Actinomycetes colonies (firm consistency, powdery or chalky

appearance) were categorized according to the release of brownish pigments into the agar as either actinomycetes$^{(+)}$ (pigment positive) or actinomycetes$^{(-)}$ (pigment negative). Very small, undifferentiable colonies were assigned to as "Pinpoints" [53].

Data analysis

Means based on six, eight, or twelve replicates for each treatment were compared by analysis of variance (ANOVA) followed by a Student-t test (P<0.05) or a Student-Newman-Keuls Test (P<0.05 or 0.1) using COSTAT 5.0 (CoHort Software, Minneapolis, MN, USA).

Results

Plant yields

Petrol hydrocarbons in soil (1517 mg TPHs kg^{-1}) have affected plant dry matter yields during 95 days (Fig. 1). Shoot yields had decreased significantly (P<0.05) for ryegrass by 38.9% and for vetch by 49.8% and root yields were reduced (P<0.05) for ryegrass by 52.6%. Still, ryegrass had the most massive root system of all three crops in contaminated soil.

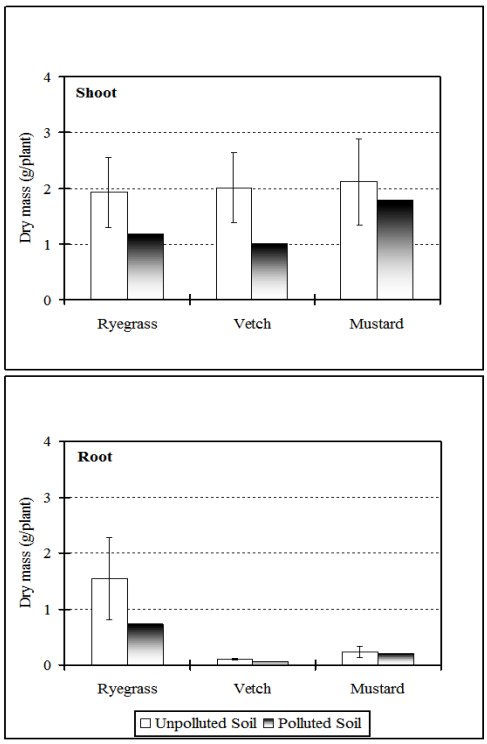

Fig. 1. Shoot and root dry matter yields in petrol hydrocarbon-contaminated soil (1517 mg TPHs kg^{-1}) after 95 days of crop growth in the greenhouse. The error bars indicate the least significant difference (LSD) (P<0,05; n=6)

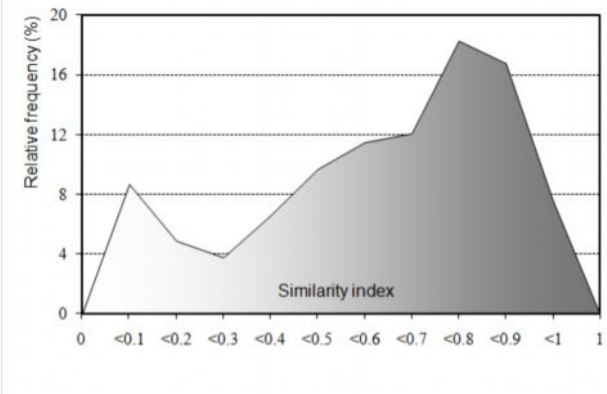

Fig. 2. Relative frequency distribution of MIS® similarity indices for all FAME-identified soil and rhizosphere bacteria (920 isolates)

Soil and rhizosphere bacterial community

Abundance and composition of the culturable soil and rhizosphere bacteria community were examined after two and eight weeks into the experiment. At 15 days, contaminated soil contained as much as 18-fold more bacteria than unpolluted soil (Table 1). Juvenile mustard and vetch raised the number of total bacteria in the root zone in unpolluted soil (R/S ratios of 1.8 and 8.2) whereas in contaminated soil, bacteria in the rhizospheres of all three crops were less abundant as compared to unplanted soil (R/S ratios between 0.3 and 0.5) (Table 1). After eight weeks, total bacteria counts in contaminated and pristine soil were alike and most numerous in the rhizospheres (Table 1). Bacteria were particularly abundant in the root zones of flowering mustard and vetch reaching 11 to 30-fold the numbers of unplanted soil.

Table 1

Number of culturable bacteria in pristine and petrolhydrocarbon (PHC)-contaminated fallow and rhizosphere soil

Contaminants	*Plant*	*Total counts (10^6 cfu g^{-1})*		*R/S ratio*[a]		*Colony types (cmt*[b] *plate)*	
		2 weeks[c]	*8 weeks*[d]	*2 weeks*	*8 weeks*	*2 weeks*	*8 weeks*
None	None	32 Ba[e]	18 Ba	NA[1]	NA	9 Aa	7 Bb
	Mustard	58 Bb	545 Aa	1,8 Bb	30,3 Aa	9 Aa	11 Aa
	Vetch	261 Aa	529 Aa	8,2 Ab	29,4 Aa	7 Aa	9 Ba
	Ryegrass	12 Bb	214 Ba	0,4 Bb	11,9 Ba	6 Ab	8 Ba
PHCs[2]	None	591 Aa	45 Cb	NA	NA	9 ABa	10 Ba
	Mustard	275 Bb	488 Aa	0,5 Ab	10,8 Aa	11 Aa	13 Aa
	Vetch	211 Cb	436 Aa	0,4 Bb	9,7 Aa	7 BCb	10 Ba
	Ryegrass	156 Cb	249 Ba	0,3 Bb	5,5 Ba	7 Cb	10 Ba

[a] R/S ratio – rhizosphere to soil ratio of total bacteria counts.
[b] cmt – colony morphotypes as obtained from original soil dilution agar plates.
[c] 2 weeks – sampling after 15 days of plant growth.
[d] 8 weeks – sampling after 57 days (mustard), 61 days (vetch), or 60 days (ryegrass, unplanted soil) of plant growth.
[e] Values in a column for either contamination level that are followed by different capital letters and values in a row for either parameter that are followed by different lowercase letters are statistically different ($P<0,05$, $n=8$).
[1] NA – not applicable.
[2] PHCs – petrolhydrocarbons.

A total of 1413 representative colony morphotypes were isolated from the original soil dilution plates and 65.2% of the isolates could be taxonomically classified by FAME analysis. Of all FAME-identified isolates, 66.3% matched a known organism in the MIS® database at similarity indices ≥ 0.5 and 82.6% had matches with a similarity index ≥ 0.3 (Fig. 2).

The bacterial community diversity was similar in contaminated vs. pristine soil but the species composition has differed (Fig. 3). At fifteen days, a total of 66 classified bacteria (including actinomycetes$^{(+)/(-)}$) were isolated from polluted compared to 69 from pristine soil. Most notably, *Bacillus* spp. were much less common in PHC-polluted soil (4 species) than in uncontaminated soil (16 species) (Fig. 3). Actinomycetes$^{(+)/(-)}$ colonies were not isolated at all from polluted soil, which, however, harbored various plant pathogens (*Acidovorax avenae*, *Clavibacter michiganense*, *Ps. marginalis*, *Ps. syringae*, *Ps. viridiflava*, *Rathayibacter tritici*) and Risk Group 2 human pathogens (BGCh, 2002) (*Acinetobacter junii*, *Actinobacillus lignieresii*, *Alcaligenes xylosoxydans*, *Bordetella bronchiseptica*, *Bordetella parapertussis*, *Enterococcus faecium*, *Kluyvera ascorbata*, *Moraxella catarrhalis*, *Ochrobactrum anthropi*, *Yersinia pseudotuberculosis*) not found in pristine soil. Particularly abundant in PHC soil were known petrol hydrocarbon degraders such as *Alcaligenes piechaudii*, *Ps. putida*, and *Stenotrophomonas maltophilia* (Table 2). After eight weeks, 75 bacteria species were identified in polluted compared to 80 in pristine soil (Fig. 3). Interestingly, the bacilli diversity has become similar in polluted and pristine soil and actinomycetes$^{(+)/(-)}$ have also appeared in PHC soil. Potential petrol hydrocarbon degraders such as *Flavobacterium johnsoniae*, *Ps. fluorescens*, *Ps. putida*, *Ps. stutzeri*, *Ps. syringae*, and *Vibrio furnissii* have still occurred only or in particular great numbers in contaminated soil (Fig. 3, Table 2).

Table 2

Abundance (10^6 cfu g^{-1}) of common bacteria populations in petrolhydrocarbon (PHC)-contaminated fallow and rhizosphere soil

Bacteria	*Plant*							
	2 week[a]				*8 week*[b]			
	None	*Mustard*	*Vetch*	*Ryegrass*	*None*	*Mustard*	*Vetch*	*Ryegrass*
Acidovorax avenae	1a[c]	0a	0a	0a	0a	6a	11a	0a
Alcaligenes piechaudii	0a	6a	26a	2a	0a	0a	5a	0a
Bordetella parapertussis	18a	0a	0a	0a	0	0	0	0
Burkholderia cepacia	0	0	0	0	0a	5a	0a	0a
Enterobacter intermedius	0	0	0	0	0a	0a	13a	0a
Flavobacterium johnsoniae	1b	7ab	13a	0b	0b	8b	34a	5b
Microbacterium esteraromaticum	0a	13a	0a	0a	1a	4a	0a	4a
Nesterenkonia halobia	0a	0a	0a	1a	0a	1a	6a	3a
Pseudoalteromonas haloplanktis	0	0	0	0	0a	3a	1a	0a
Pseudomonas balearica	0	0	0	0	0a	3a	1a	0a
Pseudomonas chlororaphis	2b	12b	0b	1b	0	0	0	0
Pseudomonas fluorescens	3ab	8a	4ab	0b	2a	11a	9a	0a
Pseudomonas putida	13b	46a	30ab	36ab	0b	31a	31a	1b
Pseudomonas stutzeri	0	0	0	0	0b	0b	11a	0b
Pseudomonas syringae	0a	2a	7a	0a	0a	4a	6a	0a
Rahnella aquatilis	0b	4b	13a	0b	0	0	0	0
Serratia liquefaciens	13a	0a	0a	0a	0	0	0	0
Serratia plymuthica	0b	1b	19a	0b	0	0	0	0
Sphingobacterium spiritivorum	9a	2a	1a	0a	0a	3a	13a	3a
Stenotrophomonas maltophilia	28ab	44a	18ab	3b	0a	3a	0a	0a
Vibrio fur nissii	0	0	0	0	0a	4a	0a	0a
Yersinia pseudotuberculosis	6a	2a	2a	0a	0b	4b	30a	1b
Actinomycetes$^{(+)}$	0	0	0	0	0b	3a	1b	0b
Actinomycetes$^{(-)}$	0	0	0	0	0b	3a	0b	0b

[a] 2 weeks – sampling after 15 days of plant growth.
[b] 8 weeks – sampling after 57 days (mustard), 61 days (vetch), or 60 days (ryegrass, unplanted soil) of plant growth.
[c] Means in rows for either sampling date that are followed by different lowercase letters are statistically different (P<0,10). Means were obtained from 12 replicates (2 weeks) and from 8 replicates (8 weeks). Means in rows for either sampling date that are not followed by letters were not comparable by ANOVA (no replicate values >0).

Occurrence and frequency of bacteria species in soil also depended on the crops. Most colony morphotypes were detected on agar plates from mustard rhizosphere samples (9 to 13 cmt plate^{-1}) and mainly from polluted soil after eight weeks (Table 1). Important hydrocarbon-degraders, including *Alcaligenes piechaudii*, *Flavobacterium johnsoniae*, *Pseudomonas* spp., and *Vibrio furnissii* were particularly promoted in the contaminated root zones of mustard and vetch (Table 2). Interestingly, the appearance of actinomycetes$^{(+)/(-)}$ in PHC soil after eight weeks was confined to the rhizospheres of mustard and vetch.

Petrol hydrocarbon degradation

Initial concentrations of TPHs (1517 mg kg^{-1}) and TPAHs (71.4 mg kg^{-1}) have declined significantly (P<0.05) in all treatments during 95 days (Table 3). In unplanted soil, 68.7% of TPHs and 59% of the TPAHs had disappeared. Mustard and vetch plants have further fostered the biodegradation of petrol hydrocarbons reaching final TPH concentrations that were 15.6% and 12%, respectively, lower than in unplanted soil. Ryegrass had no beneficial effect on the removal of TPHs and none of the crops had aided the biodegradation of TPAHs in soil.

When the extents of additional biodegradation in planted soil relative to root mass are compared, it is evident that the greatest degradative activities were associated with the small root systems of vetch and mustard (Table 3). Ryegrass did not show any degradative activity for TPHs. None of the crops exerted a root activity that aided extra biodegradation of TPAHs.

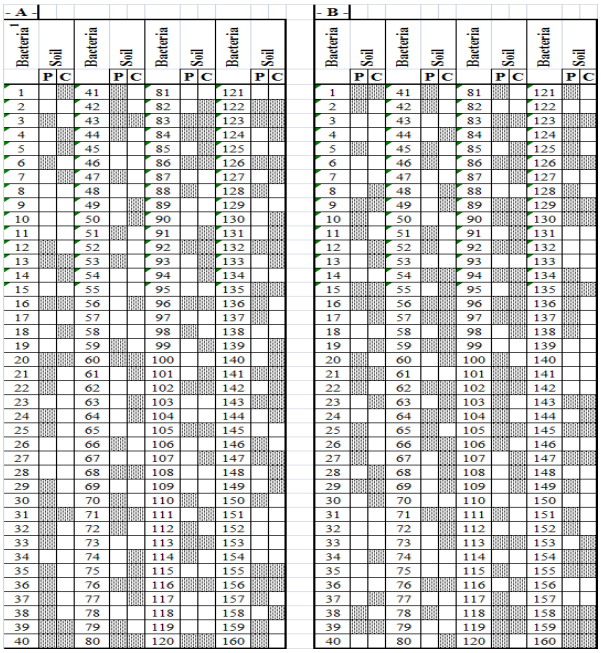

Fig. 3. Bacterial community composition in pristine (P) and petrol hydrocarbon-contaminated (C) soil (unplanted and rhizosphere) after 2 weeks (-A-) and 8 weeks (-B-) of crop growth.
Grey fields indicate the occurrence of the particular microorganisms

Bacterias: (1) *Acidovorax avenae*, (2) *Acidovorax delafieldii*, (3) *Acinetobacter baumannii*, (4) *Acinetobacter haemolyticus*, (5) *Acinetobacter junii*, (6) *Acinetobacter lwoffii*, (7) *Actinobacillus lignieresii*, (8) *Aeromonas sobria*, (9) *Agrobacterium radiobacter*, (10) *Agrobacterium rubi*, (11) *Agrobacterium tumefaciens*, (12) *Alcaligenes faecalis*, (13) *Alcaligenes piechaudii*, (14) *Alcaligenes xylosoxydans*, (15) *Amycolatopsis orientalis*, (16) *Arthrobacter agilis*, (17) *Arthrobacter atrocyaneus*, (18) *Arthrobacter aurescens*, (19) *Arthrobacter citreus*, (20) *Arthrobacter crystallopoietes*, (21) *Arthrobacter globiformis*, (22) *Arthrobacter ilicis*, (23) *Arthrobacter mysorens*, (24) *Arthrobacter nicotianae*, (25) *Arthrobacter oxydans*, (26) *Arthrobacter pascens*, (27) *Arthrobacter protophormiae/ramosus*, (28) *Azospirillum brasilense*, (29) *Bacillus cereus*, (30) *Bacillus circulans*, (31) *Bacillus coagulans*, (32) *Bacillus dipsosauri*, (33) *Bacillus flexus*, (34) *Bacillus globisporus*, (35) *Bacillus laterosporus*, (36) *Bacillus lentimorbus*, (37) *Bacillus licheniformis*, (38) *Bacillus marinus*, (39) *Bacillus megaterium*, (40) *Bacillus mycoides*, (41) *Bacillus psychrophilus*, (42) *Bacillus pumilus*, (43) *Bacillus simplex*, (44) *Bacillus sphaericus*, (45) *Bacillus subtilis*, (46) *Bacillus thuringiensis canadensis* sv., (47) *Bacillus thuringiensis kurstakii*, (48) *Bordetella avium*, (49) *Bordetella bronchiseptica*, (50) *Bordetella parapertussis*, (51) *Brevibacillus agri*, (52) *Brevibacillus centrosporus*, (53) *Brevibacterium helvolum*, (54) *Brevibacterium mcbrellneri*, (55) *Brevundimonas diminuta*, (56) *Brevundimonas vesicularis*, (57) *Burkholderia cepacia*, (58) *Cellulomonas biazotea*, (59) *Cellulomonas fimi*, (60) *Cellulomonas turbata*, (61) *Chromobacterium violaceum*, (62) *Chryseobacterium meningosepticum*, (63) *Clavibacter michiganense*, (64) *Comamonas acidovorans*, (65) *Corynebacterium ammoniagenes*, (66) *Corynebacterium aquaticum*, (67) *Curtobacterium citreum*, (68) *Curtobacterium flaccumfaciens*, (69) *Dactylosporangium fulvum*, (70) *Deinococcus erythromyxa*, (71) Deserter, (72) *Enterobacter intermedius*, (73) *Enterococcus faecalis*, (74) *Enterococcus faecium*, (75) *Flavobacterium aquatile*, (76) *Flavobacterium johnsonae*, (77) *Flavobacterium resinovorum*, (78) *Gluconobacter asaii*, (79) *Gordona bronchialis*, (80) *Hafnia alvei*, (81) *Hydrogenophaga pseudoflava*, (82) *Kluyvera ascorbata*, (83) *Kocuria kristinae*, (84) *Kocuria rosea*, (85) *Kocuria varians*, (86) *Kytococcus sedentarius*, (87) *Leuconostoc mesenteroides dextranicum*, (88) *Methylobacterium extorquens*, (89) *Methylobacterium mesophilicum*, (90) *Methylobacterium organophilum*, (91) *Microbacterium barkeri*, (92) *Microbacterium esteraromaticum*, (93) *Microbacterium lacticum*, (94) *Microbacterium liquefaciens*, (95) *Microbacterium saperdae*, (96) *Micrococcus luteus*, (97) *Micrococcus lylae*, (98) *Micromonospora carbonacea*, (99) *Moraxella catarrhalis*, (100) *Myroides odoratus*, (101) *Nesterenkonia halobia*, (102) No match, (103) *Nocardia brasiliensis* , (104) *Nocardia carnea*, (105) *Nocardia globerula*, (106) *Nocardioides albus*, (107) *Ochrobactrum anthropi*, (108) *Paenibacillus apiarius*, (109) *Paenibacillus azotofixans*, (110) *Paenibacillus gordonae*, (111) *Paenibacillus macerans*, (112) *Paenibacillus pabuli*, (113) *Paenibacillus polymyxa*, (114) *Pantoea agglomerans*, (115) *Paracoccus denitrificans*, (116) *Pedobacter heparinus*, (117) *Phyllobacterium rubiacearum*, (118) *Pseudoalteromonas haloplanktis*, (119) *Pseudomonas balearica*, (120) *Pseudomonas chlororaphis*, (121) *Pseudomonas corrugata*, (122) *Pseudomonas flectens*, (123) *Pseudomonas fluorescens*, (124) *Pseudomonas marginalis*, (125) *Pseudomonas mendocina*, (126) *Pseudomonas putida*, (127) *Pseudomonas saccharophila*, (128) *Pseudomonas savastanoi* pv. *fraxinus*, (129) *Pseudomonas stutzeri*, (130) *Pseudomonas syringae*, (131) *Pseudomonas viridiflava*, (132) *Rahnella aquatilis*, (133) *Rathayibacter tritici*, (134) *Rhodobacter capsulatus*, (135) *Rhodococcus erythropolis*, (136) *Rhodococcus fascians*, (137) *Rhodococcus globerulus*, (138) *Rhodococcus*

rhodochrous, (139) *Roseomonas* group** *Ochrobactrum*, (140) *Serratia liquefaciens*, (141) *Serratia plymuthica*, (142) *Sphingobacterium multivorum*, (143) *Sphingobacterium spiritivorum*, (144) *Sphingomonas macrogoltabidus*, (145) *Staphylococcus hominis*, (146) *Staphylococcus lugdunensis*, (147) *Stenotrophomonas maltophilia*, (148) *Streptococcus sanguis*, (149) *Streptoverticillium reticulum*, (150) *Tsukamurella paurometabolum*, (151) *Tsukamurella wratislaviensis*, (152) *Variovorax paradoxus*, (153) *Vibrio furnissii*, (154) *Xanthobacter agilis*, (155) *Xanthomonas axonopodis*, (156) *Xenorhabdus nematophilus*, (157) *Yersinia enterocolitica*, (158) *Yersinia pseudotuberculosis*, (159).

Petrol hydrocarbon degradation

Initial concentrations of TPHs (1517 mg kg^{-1}) and TPAHs (71.4 mg kg^{-1}) have declined significantly (P<0.05) in all treatments during 95 days (Table 3). In unplanted soil, 68.7% of TPHs and 59% of the TPAHs had disappeared. Mustard and vetch plants have further fostered the biodegradation of petrol hydrocarbons reaching final TPH concentrations that were 15.6% and 12%, respectively, lower than in unplanted soil. Ryegrass had no beneficial effect on the removal of TPHs and none of the crops had aided the biodegradation of TPAHs in soil.

When the extents of additional biodegradation in planted soil relative to root mass are compared, it is evident that the greatest degradative activities were associated with the small root systems of vetch and mustard (Table 3). Ryegrass did not show any degradative activity for TPHs. None of the crops exerted a root activity that aided extra biodegradation of TPAHs.

Table 3

Concentration of TPHs and TPAHs in unplanted petrolhydrocarbon (PHC)-contaminated soil and PHC soil planted to field crops

Plant	*Concentration in soil (mg kg^{-1})*				*Root wt. (g pot^{-1})*	*Degradative activity (mg g^{-1} root)*	
	TPHs[a]		*TPAHs*[b]			*TPHs*	*TPAHs*
	0 days	*95 days*	*0 days*	*95 days*	*95 days*	*95 days*	*95 days*
None	1517a	475ABb[c]	71,4a	29,3Ab	NA[d]	NA	NA
Mustard	1517a	401Bb	71,4a	29,4Ab	0,85 B	+100 B[e]	-0,1 A[f]
Vetch	1517a	418Bb	71,4a	30,5Ab	0,26 B	+259 A	-5,9 A
Ryegrass	1517a	541Ab	71,4a	30,7Ab	3,67 A	-19 C	-0,4 A

[a] TPHs – total petrolhydrocarbons.

[b] TPAHs – total of 16 US EPA priority polycyclic aromatic hydrocarbons.

[c] Values in a column that are followed by different capital letters and values in a row for either contaminant fraction that are followed by different lowercase letters are statistically different (P<0,05, n=6).

[d] NA – not applicable.

[e] (+) – plant-promoted degradation.

[f] (-) – plant-inhibited degradation.

Discussion

Ryegrass, vetch, and mustard were compared in the greenhouse for their capabilities to enhance the biodegradation of PHCs in soil from a former gas plant site. All three field crops have grown in the soil that contained 1517 mg TPHs, including 71.4 mg TPAHs per kg of dry soil. Still, plant vigor (photosynthetic activity) was depressed by PHCs (H.-H. Liste, 2003, *Habilitation* thesis) and shoot and root dry matter yields were reduced by more than 50% for ryegrass and vetch.

Toxicity of light petrol constituents (e.g. octane, BTEX) to plants and also unfavorably changed chemical, physical, and biological soil conditions can affect plant growth [36]. Poor plant growth in hydrocarbon-contaminated soils is often the result from severe water repellency that affects the water regime in soil [27; 37]. Phytosanitary aspects also may have contributed to the reduced crop growth in PHC-contaminated soil. Several *Bacillus* spp. and actinomycetes had vanished from PHC soil and these microbes were linked to plant disease suppression [49]. Correspondingly, plant pathogens, e.g. *Clavibacter michiganense* and *Pseudomonas syringae*, were more abundant in the polluted than in the pristine rhizosphere of young plants.

Despite plant growth depression, PHCs were biodegraded more rapidly in planted soil. Within 95 days, 73.6% or 72.4% of the initial 1517 mg TPH kg^{-1} have disappeared from soil planted to mustard or vetch as compared with 68.7% from unplanted soil. This is consistent with results from numerous laboratory, greenhouse, and field trials, which provide persuasive evidence that the roots of plants can aid soil bioremediation [14; 29; 30; 40].

The ability to accelerate petrol hydrocarbon degradation in soil has differed among the crops. The PHC removal during 95 day was greatest for mustard while vetch had the highest degradative activity. No remedial effect was seen for ryegrass despite its large root mass. It has repeatedly been shown that the potential to ameliorate contaminated soils differs among species and even varieties and no correlation exists between root mass and a plants capacity to foster pollutant degradation [28; 34; 56].

Plant physiological traits, namely rhizodeposition seem very important for pollutant degradation. Rhizodeposits can supply nitrogen or oxygen to microorganisms in contaminated soils that are often deficient of these elements [42], stimulate phosphate-solubilizing microbes and hence phosphorous availability [12], may enhance contaminant bioavailability [54], induce pollutant degradation pathways [24], or fortuitously select microbes that metabolize or cometabolize particular organic compounds [15]. Interestingly, mustard and vetch in particular have promoted the proliferation of bacteria, including *Alcaligenes piechaudii*, *Flavobacterium johnsoniae*, *Ps. fluorescens*, *Ps. putida*, *Ps. stutzeri*, *Ps. syringae*, *Stenotrophomonas maltophilia*, and *Vibrio furnissii* that are common and efficient degraders of aliphatic and aromatic hydrocarbons in soil [8; 46; 50; 55]. Besides, vetch may have boosted the degradation of PHCs by symbiotic fixation of atmospheric nitrogen even though the number of root nodules was lower in contaminated soil. Legumes can further enhance the clean-up of soils defiled with petrol hydrocarbons because these soils are often nitrogen deficient [23; 42–43].

After all, the promotion by plants of PHC biodegradation in soil was only moderate. Interestingly, in this regard, the number of bacteria in the rhizosphere of juvenile crops was not enhanced, which is important to note, because greater numbers and activities of microbes in the rhizosphere are considered fundamental to a plant-fostered biodegradation of organic contaminants in soil [3; 40]. This absence of an early rhizosphere effect in PHC soil was presumably the result of surplus carbon from PHCs that markedly promoted bacteria proliferation in polluted vs. pristine soil and left root-exuded carbon without the typical stimulation effect on microbial counts. Petrol hydrocarbons contain a variety of easy degradable and available compounds, including BTEX and alkanes that have boosted microbial growth and metabolism while being degraded rapidly in soil even without plants [18; 31]. Weeks later, bacteria in PHC soil were much more abundant in the rhizosphere. Easy available PHC fractions had likely ceased in polluted soil and, therefore, microorganisms became carbon limited as common in soils and only root deposits remained a major source of surplus carbon for increased microbial growth in the root zone. This delayed rhizosphere effect has eventually accelerated the degradation of residual PHCs, but 95 days were obviously too short for more decisive plant effects.

Presumably, easy available portions of organic soil contaminants must be consumed first by soil microorganisms before the remedial benefits of the rhizosphere come into full effect. As such, a more rapid depletion of readily available PHC fractions in polluted soil planted to mustard and vetch as indicated by the early appearance of actinomycetes in their root zones after eight weeks may have contributed to the more pronounced beneficial effects of both crop species on overall TPH removal. Actinomycetes only become prominent in soil when easy available organics have diminished [1] and these microorganisms play a key role in the mineralization of the more resistant and less available portions of petrol hydrocarbons such as PAHs [22].

Moreover, the stage of plant development is important for phytoremediation effects. Bacterial growth and metabolism and hence the biodegradation of organics in the rhizosphere increase gradually and often peak after a few months of plant growth [25]. The rhizosphere effect on bacterial numbers was greater for blooming than for juvenile crops in this study. Mature plants enhance the extra break down of organic matter in soil more strongly than younger plants [25], partly because young plants also need time to develop a dense root system that is prerequisite for exposing a maximum of contaminated soil to remedial rhizosphere effects. Plant-aided bioremediation of soils just takes a considerable period of time [10].

No plant-accelerated biodegradation at all was observed for the more persistent petrol hydrocarbon constituents, the PAHs. Fifty nine percent of initial TPAH amounts disappeared from bare soil within 95 days, which suggests good bioavailability. Part of the residual 41% of US EPA-PAHs was likely to be less available to microbial attack since the contaminants were in soil for many years and increasing portions of PAHs are becoming sequestered with time in soil [2; 16]. Because plants are able to enhance mobility and availability of weathered PAHs and other POPs in soil [20; 30; 33; 54] rhizosphere effects on PAH biodegradation may only become evident when bioavailable portions have disappeared and sequestered PAH residues prevail. Also and according to the diauxie phenomenon [39], microbial PAH degradation may not be accelerated, not even by plants, until the more easily degradable PHC constituents are exhausted. Polycyclic aromatic compounds were most resistant to removal from oily soils by biological treatment [19].

Of all crops, only ryegrass has hindered PHC degradation in soil. This compares with a negative rhizosphere priming effect, which is the phenomenon of a depressed decomposition of soil organic matter in the root zone of certain plant species or at certain stages of plant development [25]. This has been attributed to a decreased microbial growth and metabolism in affected rhizospheres, e.g. due to root uptake of large amounts of nitrogen and hence N deficiency in the root zone [38]. Ryegrass takes up from soil much more nitrogen than most other crops [35]. As a possible consequence, available N was very rapidly depleted in the densely rooted grass pots causing a nitrogen limitation to microbial growth. The fewer bacteria in the rhizosphere of ryegrass as compared with mustard and vetch are indicative of this. An adequate fertilization is thus critical for positive rhizosphere priming effects and an increased break down of hydrocarbons during phytoremediation and not only with respect to nitrogen because all nutrients other than carbon are strongly limited in the root zone [21; 25; 47].

However, negative rhizosphere priming effects can turn into positive ones as plant development advances [9]. For ryegrass, a negative rhizosphere priming effect has slowly become positive after 2.5 months of growth [26]. Consequently, observation periods longer than the 95 days in this study are required to examine for plant effects on the fate of organic pollutants. Yet, the biodegradation of organics in the rhizosphere can perhaps be accelerated by preventing limitations for plant and microbial growth such as by providing sufficient amounts of nitrogen via fertilization or through cropping legumes as pure or mixed stands.

Conclusions

Crops have the potential to foster the biodegradation of petrol hydrocarbons in soil but phytoremediation effects largely correlate with plant species and age and the availability of contaminants and nutrients. As long as petrol hydrocarbons are available in quantities that substantially promote microbial growth in soil, crops are not likely to aid soil bioremediation unless they overcome nitrogen or other nutrient limitations for extra biodegradation. Only as the availability to microbes of PHCs in soil declines with time, beneficial rhizosphere effects on microbial growth will increase and so may the extra break down of PHCs in contaminated soil planted to crops. Further research, particularly on the interrelations between contaminant and nutrient availability and the biodegradation of organic pollutants in plant rhizospheres is crucial for making considerable progress in phytoremediation applications.

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