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Polyaromatic Hydrocarbon Phytoremediation Stimulated By Root Exudates

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Abstract To encourage polyaromatic hydrocarbon (PAH) degradation in a historic steel production facility, contaminated soil was amended with 10% (v/v) compost and 5% (v/v) poultry litter. Within twelve 11.89 m X 27.13 m plots, 35,000 native Michigan perennials were planted. Soil and heterotrophic bacteria were sampled over several years beneath unplanted (control), *Eupatorium perfoliatum* (Boneset), *Aster novae-angliae* (New England Aster), *Andropogon gerardii* (Big Bluestem), and *Scirpus atrovirens* (Green Bulrush). All soils were found to degrade many PAHs, in one case up to 37% of the total. Cultivable microbes from the beneath plants were recovered, and 16S rDNA sequencing was used to identify microbial species. Implicated in phytoremediation, root exudates were prepared from select plants. Exudate amino acid composition changed with increasing plant age. A shift from Met and Lys to Glu and Asn was observed in exudates obtained from Swamp Goldenrod (*Solidago patula*). In Boneset, and New England Aster, Gly and Ala comprised at least 10% the total amino acids. Besides remediating a large fraction of the soil PAHs, the majority of planted plants survived the 3-year experiment. Root secretions and indigenous microbial communities may establish beneficial relationships that promote *in situ* PAH phytoremediation.

Keywords: *bioremediation, native plant species, environmental toxicity cleanup*

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1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are environmental contaminants with cytotoxic, teratogenic and carcinogenic properties. PAHs with more than three aromatic rings are known as HMW PAHs. These toxicants are produced during the incomplete combustion of organic material through pyrolysis and pyrosynthesis, and are abundant in fossil fuels [1,2]. HMW PAHs are slightly soluble in water, are electrochemically stable and may be acutely toxic, putting at risk human and ecological health [3]. The physical and chemical properties of HMW PAHs contribute to their being persistent in the environment, in both their initial and chemically transformed forms [4]. Because of their high octanol–water partition coefficients (K_{ow}), HMW PAHs may predominate in organic phases in soil and sediments, and bioaccumulate [5].

In situ bioremediation may relieve the "cradle to grave" transition of raw materials flowing into manufactured goods, subsequently discarded in traditional landfills or on farmland [6]. Such pollutant dumping can be harmful to plants growing in those soils. Some fuel oils contain hydrocarbons that inhibit plant growth as well as seed germination [7]. PAHs and other organic pollutants derived from pesticides, coal or other fuel combustion, or from sewage may persist in soils and contaminate the plants grazed upon by livestock [8,9,10]. Poor plant growth noted in some contaminated soils can be attributed to acidification and nutrient depletion of soils. Using alder plants and root inoculation of N_2-fixing bacteria such as *Frankia* and/or ectomycorrhizal fungi, mining waste soil pH was effectively adjusted to 7.5 [11]. *Novosphingobium* sp. are found in soils, and can effectively detoxify contaminants, greatly improving plant growth [12]. Nitrogen availability is also a key limitation for plant growth on contaminated sites. The addition of N-fertilizer was implicated in increasing sugars, sugar alcohols, and phenolics in root exudates, thereby enhancing the number and diversity of biodegrading rhizosphere bacteria [13]. Using select plants, soil detoxification with phytoremediation can lead to the subsequent introduction and growth of new plant and microbial species [14,15,16].

While some plant species secrete enzymes capable of transforming aromatic pollutants [17], it is widely believed plants more commonly stimulate changes in microbial populations and promote PAH metabolism in situ, [16,18,19,20]. Microbial population titer and composition all are thought to be potential targets affected by root exudates [20]. Laboratory studies have shown that roots release nutrients and organic compounds that may facilitate microbial growth and PAH biodegradation [15,17,21,22]. Exuded from plant roots, plant metabolites such as sugars, amino acids, phenolic and terpenoid compounds may all contribute to bacterial degradation of organic contaminants

[16,23,24,25]. Support for root exudate ingredients positively facilitating degradation comes from exogenous addition of exudate contents such as hydroxycinnamic acids, flavone, morin, and 3-hydroxyflavone, which were reported to improve PAH mineralization [20,26]. There is also evidence that some root exudates can contain acetate, lactate, pyruvate, glutamate, and glucose, which in turn can repress phenanthrene destruction by a catabolite repression process [27,28].

The goal of this study was to examine the rhizosphere population from phytoremediating plants, and note their responses to root exudates during PAH degradation. PAH-impacted soils were planted in mono-specific and multi-species sub-plots with a variety of Michigan native species. Root exudates from different plant sources promoted several microbial isolates to grow faster, compared to un-supplemented cultures and at somewhat differing concentrations, dependent on the donor plant genus and the age of the plant providing the exudates. Plant exudates varied in amino acid content, depending on the plant species and the age of that plant when grown in hydroponics. Microbial populations changed in this experiment [16], and most of plants initially planted in the PAH contaminated soil did survive. Destruction of soil PAHs did not negatively affect continued plant perseverance. PAH contaminant outcome in vegetated plots was effectively remediated compared to the non-vegetated soils.

2. Materials and Methods

2.1. Materials

Polyaromatic hydrocarbon (PAH) tainted soils were obtained from the Coke Oven Facility at the Ford Rouge Manufacturing Complex (Dearborn, MI). Coke Oven. Composted yard litter (5% v/v; Ypsilanti County, MI) and composted poultry manure (5% v/v; Herbruck's Poultry Farm, Saranac, MI) were mixed with contaminated soil to a final volume of approximately 200 cubic yards of Rouge Soil Mix. Each of 10 39' X 35' plots was subdivided into mono- and multi-species planting. Selected native Michigan plants were obtained from Wildtype Nursery (Mason, MI) and included grasses, herbaceous dicots, and woody shrubs.

2.1.1. Soils

Soil samples were taken at two years after site planting and placed in glass jars, chilled to 4C and sent overnight to Kemron Environmental Services (Matrietta, OH). Extraction, PAH fractionation, and determination followed EPA 8270C guidelines. PAH detection was facilitated with GC/MS and the appropriate PAH standards.

2.1.2. Environmental Bacterial Isolates

Microbial isolation occurred within 48 h after soil sampling. One gram (fresh weigh) of soil was suspended in 10 ml 0.1% (w/v) sodium pyrophosphate (pH 7), shaken for 30 m, and the aqueous fraction serially diluted in 0.1% sodium pyrophosphate. From separate dilutions, 100µl was placed on a YEPG agar plate in triplicate, the solution distributed, and the plate incubated in the dark at room temperature for seven days. Two complete sets of dilutions and plates were made. YEPG agar is comprised per litre of 0.05 g yeast extract, 0.05 g ammonium nitrate, 0.5 g Bacto peptone, 0.25 g glucose, 15.0 g Bacto agar (Difco, Detroit, Michigan). Individual colonies were re-streaked and grown for an additional week and were subsequently re-streaked a second time and recultured to confirm cultures from a single isolate colony.

2.1.3. Bacterial DNA

Microbial DNA was extracted from 2 day old cultures using 0.1µm glass beads (BioSpec Products, Inc. http://www.biospec.com) and 500µl of extraction buffer (100mM Tris-HCl [pH 8], 100mM EDTA and 1.5M NaCl, followed by a PEG precipitation [29]. PCR conditions of 16S rDNA employed the 8F and 926R primers as described [30]. DNA sequence was edited (4Peaks, http://mekentosj.com/4peaks), and sequences used to identify the genus of the microbe (Ribosomal Database for Microbes at Michigan State University. (http://rdp.cme.msu.edu/).

2.1.4. PAH Enumeration

The Wrenn and Venosa procedure [31,32] was used with modifications [25] to enumerate PAH degrading bacteria. Wells of a 96 well plate were coated with 10 µl of 10 mg^{-1}l phenanthrene, 1 mg^{-1}l dibenzothiophene and 1 mg^{-1}l of fluorene dissolved in pentane or mixed hexanes. After drying, 190 µl YEPG liquid medium with or without plant exudate, and 10 µl of a 2 day YEPG incubated bacterial culture were added to each well. The plate incubated in the dark for three weeks. Three weeks later, the resultant medium color and intensity was judged on a scale of 0 (clear), 1 (pink/tan), 2 (red/light brown) or 3 (dark red/dark brown).

2.1.5. Exudates

Exudates were recovered from plants grown in the laboratory in 1/2X Hoaglands nutrient solution [33], and under 16h light 150-200 µEinstein m^{-1} sec^{-2} at 20-24C. For exudate recovery, plants anchored to a light tight lid were removed; the roots rinsed with tap water, and then placed in 50% DI and 50% tap water for 2 h. Afterwards, the roots were returned to fresh nutrient solution, and the water/exudate sample was freeze dried. Exudate samples were subsequently re-suspended in sterile DI water (0.5ml/plant), filter sterilized using a 0.2µm filter, and frozen at -80C. Amino acid analysis was performed at the University of Michigan, Protein Structure Facility (Dr. Henriette A. Remmer).

Healthy pre-flowering adult plants were either obtained from seeds or removed from the field and grown for at least 4 weeks in hydroponic culture. Use of hydroponics and light tight lids for the plants greatly facilitated the recovery of root secretions. Upon observation after exudate collection, there was no change in the color of the roots. These same roots continued to grow, produce side roots, and elongate throughout the experiment. In Swamp Goldenrod, an odor of horseradish was conspicuous in the root tissues immersed in hydroponics.

3. Results

3.1. Exudate Influence on Microbial Growth

Soil microbial colonies were recovered during the first 2 years of this experiment. Several randomly selected microbes beneath the NE Aster plants, or unplanted soil, were recovered and screened for PAH degradation. In the presence or absence of various exudates, growth of the respective microbes was followed by changes in optical density at 600nm in a Bio Rad MiniPlate Reader (BioRad Laboratories, Hercules, CA) (Figure 1). All three independent environmental isolates did positively respond to root exudate addition. This is a representative experiment of several; most that showed exudates enhanced growth of individual isolates.

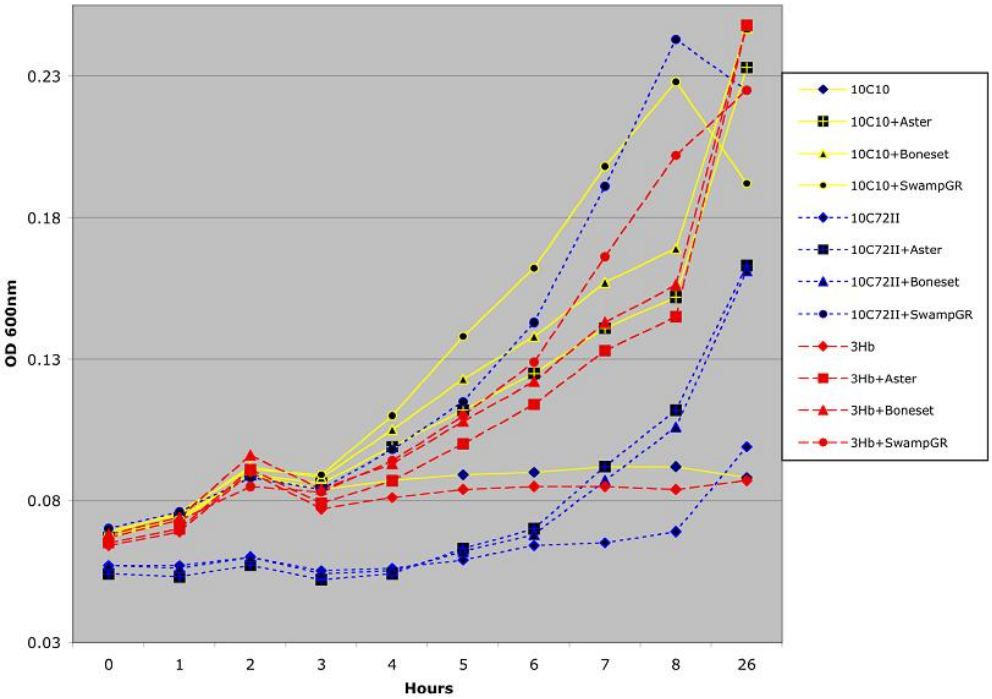

Figure 1. The influence of 0.35% v/v exudates from NE Aster, Boneset, or Swamp Goldenrod (*Solidago patula*), compared to no added exudates on the growth of two randomly selected PAH degrader microbes isolated from control soil (i.e. unplanted) or soil beneath NE Aster plants. Microbial growth was assessed in liquid YEPG medium as the increase in OD 600nm. Microbes for the test were randomly chosen from a consortium of purified environmental isolates

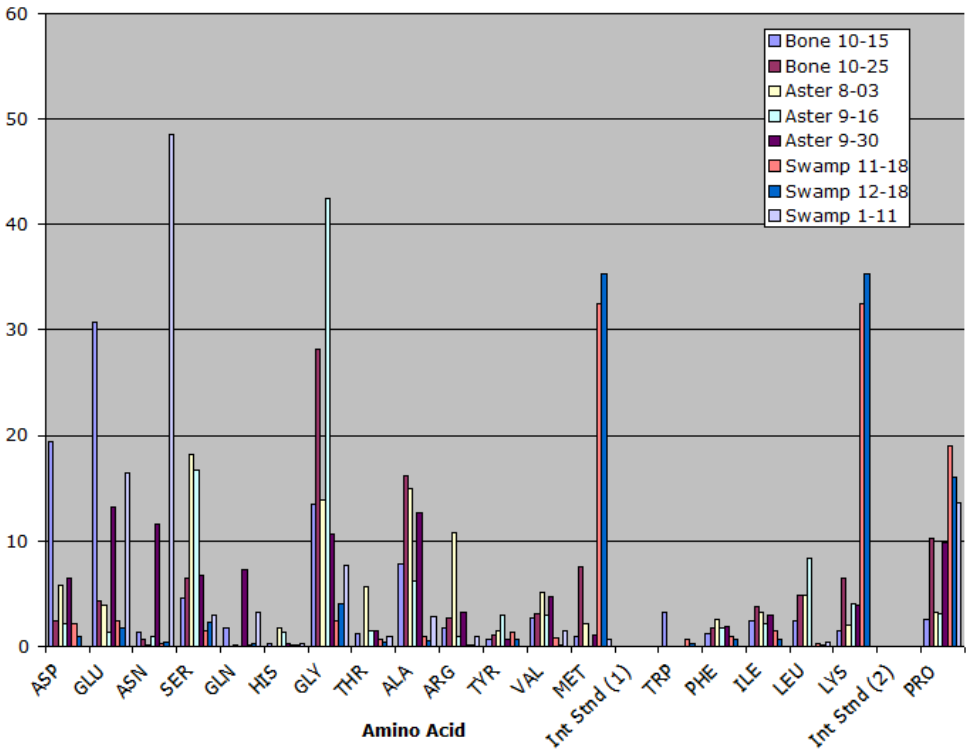

Figure 2. Amino acid composition expressed as a % of the total. Total amino acids recovered per exudates isolation was: Bone; Boneset, Aster; New England Aster, and Swamp; Swamp Goldenrod

3.2. Amino Acid Composition of Root Exudate

Acknowledging that root exudates from plants may differ in field and lab conditions, we found the most mature exudates of Swamp Goldenrod contained 26 and 17 fold greater total amino acids (nmol·L^{-1}·(mL exudate)$^{-1}$ compared to Boneset and New England Aster, respectively [25]. Within a given plant species, relative changes in the exudate amino acids were observed (Figure 2). Boneset and New England Aster tended to increase their proline percentage during hydroponic culture: 3.9 and 3.0 fold respectively. Swamp Goldenrod proline percentage declined from 2 weeks and 10 weeks: - 0.7 fold. All Boneset and Aster exudates contained greater than 10% glycine and alanine, whereas Swamp Goldenrod was a bit different, containing 48.5% asparagine, 16.6% glutamic acid.

3.3. PAH Degrader Enumeration

At least 100 environmental isolates were purified using single colony plating. Upon screening for PAH degradation enumeration, about 2% of the microbial isolates were recorded as positive for PAH degradation (Table 1). When 0.35% Swamp Goldenrod exudate was added, the % degraders increased 10 fold, to 20% of all isolates on average (Table 1).

3.4. Soil PAH Levels

Soil samples were recovered from the phytoremediation site after 2 years. They were immediately shipped to a commercial environmental laboratory for analysis (Kemron Environmental Services (Marietta, OH). PAH levels are shown in Table 2. In comparison, a single sample from 2002, prior to the site construction, was analyzed and shown below (Original).

Analysis of the PAH content of soils indicated that the level of contamination (at least from one soil sample (Original) was high. Upon dilution with other site soils and the soil amendments, as well as soil mixing, control soils were nearly 270 ppm in 2 years. All three planted soil samples lost between 20-37% of the control soil total PAH in 2 years.

Table 1. Percentage of isolated bacteria from the field site that were positive for PAH degradation after 3 weeks (31). Data is from a subset of samples (N =35) from 2004. + exudate = 0.35% (v/v) Swamp Goldenrod exudate in YEPG medium. The - exudate is identical except lacking root exudate

Plant Plot	Positives (% colonies + exudate)	Positives (% colonies - exudate)
New England Aster/Blue Stem	32	5
Bonest	29	3
New England Aster	10	0
Green Bulrush	10	0
Control	20	3

Table 2. PAH levels were reduced in some planted plots after 2 years of growth. The table represents the mean of two soil samples

PAH	Bulrush	NE Aster	NE Aster/Bluestem	Control	Original
Naphthalene	29900	31400	15500	30700	46100
Acenaphthylene	5450	4580	5600	7870	35300
Acenaphthene	1200	1260	407	1220	2570
Fluorene	2940	2800	1860	4030	15500
Phenanthrene	21200	17000	21100	29500	89400
Anthracene	5990	4880	3810	8880	34600
Fluoranthene	27300	19400	21600	39000	202000
Pyrene	22200	16800	21800	32100	172000
Benzo(a)anthracene	12400	10300	17100	18000	115000
Chrysene	10900	8620	15700	14900	93700
Benzo(b)fluoranthene	17100	13700	24300	23500	162000
Benzo(k)fluoranthene	5260	4620	8660	8480	62100
Benzo(a)pyrene	13800	11600	18400	20600	140000
Indeno(1,2,3-cd)pyrene	8150	6780	12700	12000	86400
Dibenzo(a,h)anthracene	0	0	0	0	0
Benzo(g,h,i)perylene	9870	8350	15700	14600	106000
1-Methylnaphthalene	3010	3530	4790	2210	4760
2-Methylnaphthalene	4770	5530	7220	3680	6820
Total PAH ppb	201440	171150	216247	271270	1374250
% PAH remaining	**74.26**	**63.09**	**79.72**	**100.00**	**506.60**

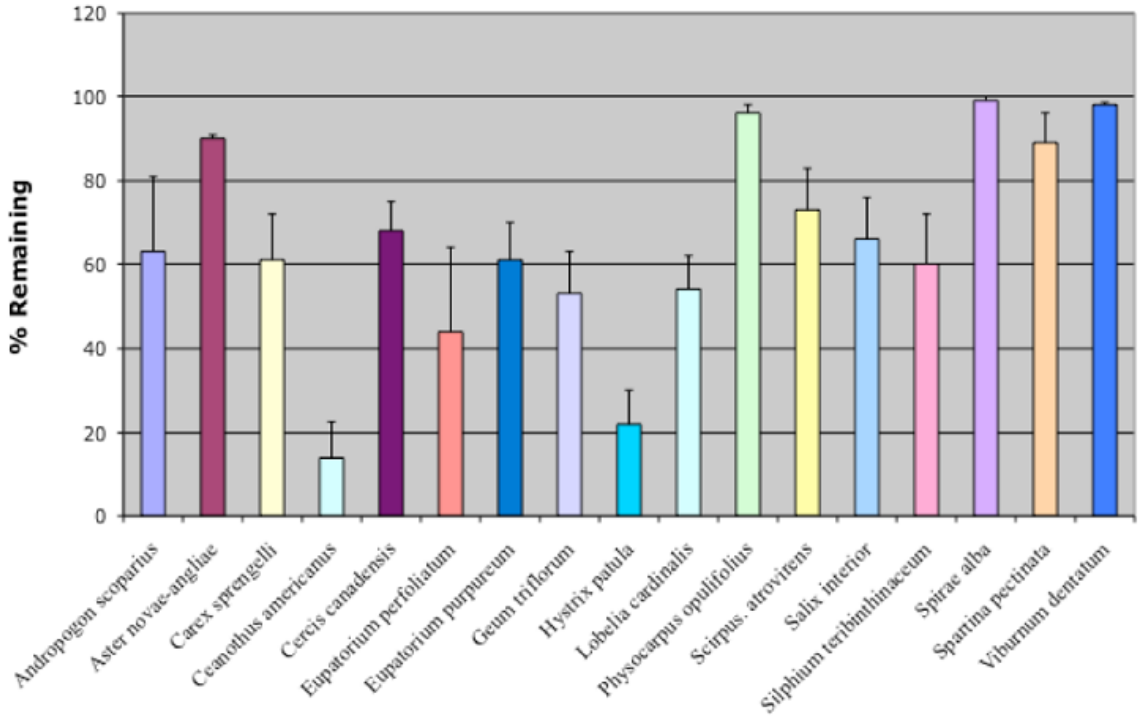

Figure 3. The remaining plant species after three years of phytoremediation. Three separate plant plots were counted per plant species and the average and standard deviation depicted above

3.5. Native Plant Species Remaining

After three years, each plot was scored for the percentage remaining plant species. The majority of plant species remained at approximately 60% (Figure 3). This information provides insight into the ability of that native species being maintained while concurrently experiencing PAH biotransformation.

4. Discussion

4.1. How Plants May Encourage PAH Loss

Given that PAHs are relatively water insoluble, they can remain for some time in soils. An emerging technology is the use of plants for promoting biochemical processes linked to environmental recovery. Either by stimulating contaminant bioavailability and/or enriching PAH degrading microbial populations (Figure 1), root exudates can significantly increase PAH degradation in rhizosphere soils [34,35], or exudates may also inhibit microbial-based bioremediation [27]. Constituents of root exudates, amino acids, flavones, and carbohydrates are likely contributors to the bio-stimulatory effects of root exudates [26,36]. Enhanced proline production is often synonymous with abiotic plant stress and heavy metals [37,38]. While Boneset and New England Aster exudates increased in proline with the age of the plants, the most effective exudate for PAH degradation (Swamp Goldenrod) saw a decline in proline levels as the plants aged (Figure 2) [25]. In this study, plant exudates produced by native Michigan plants likely encouraged the removal of polyaromatic hydrocarbons observed here is a favorable outcome for this former industrial site [14,16,19].

Plants can greatly influence the degree of remediation in situ [15,39,40]. This phenomenon is due in large part to the prospective bacterial community being supported during phytoremediation of persistent organic contaminants such as PAHs [20,41]. Here we demonstrated that some plant exudates could promote soil degradation, increasing from 2 to 20% of all microbes screened (Table 1). We establish the capability of some Michigan native plant species, with the contribution of their root exudates, can enhance PAH degradation.

4.2. How Microbes Facilitate Phytoremediation

How do root exudates influence microbes that can lead to degraded PAHs? Possibilities include improved removal of PAHs from clays and other soil constituents, or perhaps altering gene expression to present degradation enzymes these anthropogenic contaminants. This question remains difficult to address as the number of degrader microbes from the field capable of PAH degradation was not strictly correlated with PAH content remaining in soils after 2 years (Table 2). This finding may be due to only a fraction of soil bacteria that can be cultured as described [32]. As part of this question, microbial population shifts during remediation have been described [42]. It is also highly likely that the complex relationships between plant and microbial species in co-metabolism as well as direct carbon source utilization needs to be better elucidated in order to select strategic plant species that interact with specific microbes to provide the desired phytoremediation.

We analyzed a suite of selected native Michigan plant species in an experimental phytoremediation field trial. The analysis suggests these regional, perennial plant species can greatly impact PAH contaminated Brownfield facilities by reducing soil PAHs. Generally, each plant species tested may influence the number of PAH-degrading soil bacteria density. A significant fraction (30%) of microbial isolates derived from some planted treatments

possessed the metabolic capability to degrade multiple PAH substrates when co-cultured with root exudates.

5. Conclusion

Work presented here strongly suggests the importance of plant-derived exudates to promote bacterial PAH degradation *in vitro*. At least some microbes demonstrated growth stimulation with exudate treatment (Figure 1). In the future, modern molecular and ecological methods might be more effective in characterizing site-specific microbial consortia an better able to correlate exudate responsiveness and PAH degradation [43,44,45]. In spite of challenges [46], future management of beneficial plant-microbe interactions may be the key to rapid and successful in situ PAH phytoremediation strategies.

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