Three Year Field Test of a Plant Growth Promoting Rhizobacteria Enhanced Phytoremediation System at a Land Farm for Treatment of Hydrocarbon Waste

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Phytoremediation of total petroleum hydrocarbons (TPH) has the potential to be a sustainable waste management technology if it can be proven to be effective in the field. Over the past decade, our laboratory has developed a system which utilizes plant growth promoting rhizobacteria (PGPR) enhanced phytoremediation (PEP) that, following extensive greenhouse testing, was shown to be effective at remediating TPH from soils. This system consists of physical soil manipulation and plant growth following seed inoculation with PGPR. PGPR elicit biomass increases, particularly in roots, by minimizing plant stress in highly contaminated soils. Extensive development of the root system enhances degradation of contaminants by the plants and supports an active rhizosphere that effectively promotes TPH degradation by a broad microbial consortium. Following promising greenhouse trials, field tests of PEP were performed over a period of three years at a Southern Ontario site (~130 g kg^{-1} TPH) used for land farming of refinery hydrocarbon waste for many years. The low molecular weight fractions (the Canadian Council of Ministers of the Environment (CCME) fractions 1 and 2) were removed through land farming and bioremediation; the high molecular weight, recalcitrant fractions (CCME fractions 3 and 4) remained at high levels in the soil. Using PEP, we substantially remediated fractions 3 and 4, and lowered TPH from 130 g kg^{-1} to ~50 g kg^{-1} over a three year period. The amount of plant growth and extent of oil remediation were consistently enhanced by PGPR.

Introduction

The large number of petroleum products released into the environment characteristically consist of aliphatic, aromatic, heterocyclic, and asphaltene/tar hydrocarbons ranging in size from C6 to C > 50. These compounds, collectively labeled total petroleum hydrocarbons (TPH), have been classified into various fractions, where fraction 1 (F1) contains C6−C10; fraction 2 (F2), C10−C16; fraction 3 (F3), C16−C34; fraction 4 (F4), C34−C50+; and higher fractions contain compounds C > 50 (*1*). F1 and F2 are volatile or semivolatile, whereas F3 and F4 are very hydrophobic and recalcitrant to breakdown. Compounds from F3 are often highly toxic and are regulated due to their mutagenicity and carcinogenicity (*1, 2*).

Toxicity of TPH has been a motivating force in finding sustainable biological methods of remediation for these compounds. Among the many existing biological remediation approaches, strategies such as land farming or in situ bioremediation are of great interest. However, these methods are often not capable of sustaining dense growth of indigenous microorganisms nor those added to promote degradation, due to nutrient restrictions and toxicity of contaminants (*3−6*). Phytoremediation provides a viable option for efficient and cost-effective remediation of contaminated soils because adding plants to the remedial system addresses the biomass production limitations of other biological methods (*7−9*). Plants exude soluble nutrients that can be utilized as an energy source by microorganisms, and plant roots provide a substratum to increase microbial growth in the rhizosphere (*9−14*). Many plant species, grasses in particular, have extensive root systems that can generate a great deal of biomass into large volumes of soil (*15*), thus promoting an active rhizosphere, which can consume soil contaminants. Indirectly, the presence of the plants enhances degradation through changes in the soil profile induced by root growth, such as creating channels for water/air penetration, increased surface area for microbial growth and changes in pH in response to nutrient status (*11*).

Although phytoremediation has potential as a viable remediation strategy for persistent organics, several limitations hinder its widespread application in the field (*8, 9, 16*). Contaminants can affect plant photosynthesis, respiration, and metabolism resulting in low plant biomass and, subsequently, low remediation (*17*). To increase plant biomass in contaminated soils, plant growth promoting rhizobacteria (PGPR) can be used to mitigate plant stress responses, and enhance degradation of contaminants (*18−20*). Many PGPR have the ability to consume 1-amino-cyclopropane-1-carboxylic acid (ACC), a precursor to the plant stress hormone ethylene. This degradation is directly dependent on the bacterial activity of ACC deaminase (*21*). Lowered ethylene biosynthesis in plants can facilitate growth under stress conditions in highly contaminated soils (*16*). These PGPR act by alleviating contaminant induced stress, thus allowing adequate biomass accumulation for acceptable rates of remediation (*16, 19, 20*). In greenhouse studies, PGPR have proven effective for improving phytoremediation of petroleum and other contaminants (*19, 20, 22*). Although some field scale phytoremediation studies have emerged (*23*), the benefits of using PGPR in TPH remediation have not been fully validated in the field (*24, 25*).

A PGPR-enhanced phytoremediation (PEP) system was developed specifically for the removal of toxic, persistent, and recalcitrant contaminants from soil (*19, 20, 26*). In PEP, phytoremediation is performed with the addition of PGPR to increase plant biomass in contaminated soils via alleviation of plant stress by ACC deaminase activity. Based on the success of the greenhouse experiments, where PEP outperformed microorganisms alone and plants alone (*19, 20*), the objective of the research reported here was to test the efficacy of PEP in the field at an oil refinery land farm site in Sarnia, Ontario, Canada. This was carried out between April 2004 and October 2006. This particular site was chosen due to high contaminant levels, which presented a tremendous remediation challenge for PEP; to our knowledge no attempts to phytoremediate concentration as high as 130 g kg^{-1} TPH have been reported. Parameters such as plant growth and

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photosynthesis were assessed as indicators of plant health and remediation. Most remediation technologies are site specific, and successful application depends on careful experimental design and adjustments based on changes in soil and chemical properties as the remediation process progresses (*27*). Indeed, we accelerated the transition of PEP from laboratory to field trials by applying lessons learned in the laboratory and field immediately in the subsequent years of field trials reported here. Most importantly, plant growth (annual ryegrass, tall fescue, barley, and fall rye) in the field was enhanced by PGPR, remediation was commensurate with plant growth, and remediation appeared to follow first order kinetics.

Experimental Section

Site Description. Field tests were performed for three consecutive years from May 2004 at an oil refinery land farm in Sarnia, Ontario, Canada. At this site, petroleum sludge was regularly spread on the soil horizon and tilled over the last 20 years between the months of May and October. Two rectangular plots of 130 × 40 m each, i.e., site 1 and site 2, were divided into experimental plots with paired block designs. Both sites were somewhat homogeneous in TPH concentrations. Constant land farming prevented establishment of any vegetation, and plant growth had not been attempted on this site prior to the study. At the onset of the trial, TPH levels were approximately 130 g kg^{-1}, ranging from 99 to 148.5 g kg^{-1}.

Soil Remediation Using PEP. The remediation strategy consisted of physical manipulation of the soil performed through tilling, sunlight exposure (aeration/photooxidation), and plant growth with PGPR. Plants with PGPR-treated seeds are hereafter referred to as PGPR plants or as PGPR-treated plants. Plants were selected based on their suitability to the Southern Ontario climate, and their tolerance to hydrocarbon stress (*19, 20, 26*). In 2004, site 1 was planted with annual ryegrass (*Lolium perenne*) with and without PGPR treatment. Subsequently, site 1 was chosen to evaluate remediation kinetics. Temporal analysis was performed with PGPR treatment only, and there was no direct comparison between ±PGPR treatments for 2005 and 2006 for site 1. In 2005, annual ryegrass and tall fescue (*Festuca arundinacea,* var. Inferno) were planted on site 1 and fall rye (*Secale cereale*) was used to overseed in the fall. In the spring of 2006, barley (*Hordeum vulgare*) was added to the annual ryegrass/tall fescue mixture. The following plants, and plant mixtures, were used in this study at site 2: year 2 (2005), annual ryegrass, annual ryegrass and tall fescue mixture, barley and fall rye mixture; year 3 (2006), annual ryegrass and tall fescue mixture, and annual ryegrass, tall fescue and barley mixture. Seeds were bought from Ontario Seed Co., Waterloo, Ontario, Canada (annual ryegrass, tall fescue, fall rye), and from Cribit Seeds, West Montrose, Ontario, Canada (barley). Every year, ±PGPR treatments were tested on site 2. Seed planting density was approximately 300 seeds m^{-2}. Plants were allowed to grow for the entire growing season (~150 days) and were irrigated as needed. The control area consisted of soil that was not vegetated and received tilling treatments concurrently with planted sites, at the beginning of every growing season. Treatment with herbicides was not necessary to keep this area free of vegetation.

Seed Treatment with PGPR. Two PGPR *Pseudomonas* strains, UW3 and UW4 (*28*), were introduced to the rhizosphere by applying them to seeds. UW4 has previously been identified as *Pseudomonas putida* (*29*), and UW3 has been identified as *Pseudomonas* sp. UW3 has been characterized using biochemical tests, 16S rDNA sequence and other biochemical and microbiological tests and found its closest species matches to be those of nonpathogenic bacteria from genus *Pseudomonas.* Both strains are susceptible to tetracycline and kanamycin, and will not grow at 37 °C. For seed treatment, bacterial strains were grown in tryptic soy broth (30 g L^{-1}, Fisher Scientific, Ottawa, Ontario, Canada) at RT for 24 h, until they reached an absorbance of 2−3 at 600 nm. A Hege 11 liquid seed treater (Wintersteiger, Saskatoon, Saskatchewan, Canada) was used to apply PGPR to the seed. The bacterial culture was centrifigued and the pellet was resuspended in deionized water. A methyl-cellulose polymer (Sigma-Aldrich, Oakville, Ontario, Canada) coating was used to facilitate PGPR adhesion to the seeds. Seeds treated with PGPR were also treated with colorant (Color Coat Blue, Becker Underwood, Saskatoon, Saskatchewan, Canada) to distinguish treated and untreated seeds. The colorant did not affect plant growth or PGPR efficacy (data not shown).

Soil Sampling and Chemical Analysis. Soil samples (250 g) from planted and unplanted areas were collected at least three times per year, using an Edelman auger (Eijelkamp Agrisearch Equipment, Giesbeek, The Netherlands). Single grab soil samples were taken systematically, in a grid pattern in each plot to ensure complete coverage of the site. A sampling grid was established with a point taken every 10 m in all directions on the site. The sample was taken randomly within a 4 m^2 area around a given grid point to a depth of 30 cm, in areas with representative amounts of plant growth. Duplicate samples were collected regularly and analyzed to ensure accuracy of sampling and analyses, and to control for sampling anomalies. Samples were placed in glass jars, and stored at 4 °C until further analysis. Levels of TPH were determined gravimetrically. The use of ultrasonic solvent extraction was chosen for this particular site because contamination consisted mostly of the heavier petroleum fractions, F3 and F4, therefore the loss of volatile hydrocarbons was not a major concern. Briefly, air-dried soil samples (2 g) were extracted three times by ultrasonication for 50 min into a total of 20 mL of 1:1 hexane/acetone mixture (*30*). To ensure extraction efficiency (completeness) the soil was periodically extracted for a fourth time and those extracts always contained less than 5% of the TPH in the soil. As well, we compared our gravimetric data to those from certified analytical laboratories (see below); the extraction efficiency of these analytical laboratories was generally >95% and our data were on par with this data (Supporting Information (SI) Figure S3). Extracts were dried by completely evaporating the solvent under a stream of nitrogen gas. The amount of petroleum sludge was determined by weighing the dried extracts. Replicate variation was <10%. The TPH content and CCME fractions 1−4 measurements in soil samples were analyzed independently by two certified laboratories: ALS Environmental (Waterloo, Ontario, Canada) and Maxxam Analytics Inc. (Mississauga, Ontario, Canada). These analyses were performed according to standard protocols of the CCME (*31*). CCME fractions 1−4 were determined using gas chromatography with flame ionization detector and a 100% poly(dimethylsiloxane) column, following a hexane:acetone extraction (*31*). For QA/QC requirements, to ensure accuracy and efficiency of TPH extraction in our laboratory, samples with known TPH concentration were analyzed along with new field samples on an ongoing basis. Concentration for the known samples had to be within 10% of the established value for the analytical run to be considered acceptable. To further assess the accuracy of analysis, at least 15% of all soil samples were sent to the independent laboratories for analysis; split samples were sent to compare the gravimetric analysis TPH results of our laboratory to that from an analytical laboratory. Only if the data from our laboratory and the analytical laboratory were consistent was the data used. An example of this evaluation is in SI Figure S3 where the comparison yielded a straight line with a slope close to 1 and a *y*-intercept close to zero (r^2 = 0.85). Further, our

gravimetric data collected can be correlated to the F3 and F4 data obtained from the analytical laboratories (data not shown).

Plant Biomass, Plant Length, and Ground Cover Measurements. Plant samples were collected at least three times per year. Plant growth was measured in one of two ways: root and shoot length, or root and shoot biomass. Plant biomass was measured by isolating a 50 × 50 cm square of soil, 30 cm in depth, with as little disturbance of roots as possible. Soil samples were further divided into three subsamples. Plants, including roots, were isolated from the soil and washed with water to remove all soil particles adhering to the roots. Roots and shoots were separated and blotted dry to obtain fresh weight. To determine dry weight, plant samples were dried for 2 days at 40 °C in an oven and reweighed. All measurements were normalized to those of untreated plants and averaged to obtain annual plant performance in terms of root and shoot biomass (fresh and dry weight). Ground cover measurements were performed in triplicate by recording percent plant ground cover in a 1 m^2 quadrant, with a minimum three measurements taken each time.

Plant Photosynthetic Activity. Effects of hydrocarbon stress were assessed by measurements of the photosynthetic activity of plants measured by chlorophyll-*a* (Chl *a*) fluorescence induction using a pulse amplitude modulation (PAM) fluorometer (PAM-101, Walz, Effeltrich, Germany). The protocol as described in Marwood et al. (*32*) was used. Values obtained from PAM fluorescence were F_v/F_m (maximum efficiency of photosystem II (PSII)), yield (effective quantum yield of PS II under steady-state conditions), qP (coefficient of photochemical fluorescence quenching), and qN (coefficient of nonphotochemical fluorescence quenching) (*33*). Sampling was performed at least three times per year from site 2. PAM fluorometry measurements were performed in 2005 and 2006, on plants with and without PGPR treatment.

Statistical Analysis. To determine significance, ANOVA was used along with Tukey–Kramer post-test. Degradation rates were calculated by assuming first-order kinetics. Degradation curves were fitted using nonlinear regression by Systat Software (Systat Software, Point Richmond, CA) to the first-order kinetic equation given below:

$$C = C_0 \exp(-kt) \quad (1)$$

C is the TPH concentration (g kg^{-1}), *t* is time (months), C_0 is the initial TPH concentration, and *k* is the kinetic rate constant ($month^{-1}$).

Results

Overview of Experimental Results. In year 1 (2004), the complexity of the experimental design was kept to a minimum. The main goal was to determine whether plant growth was feasible on soil contaminated with TPH averaging 130 g kg^{-1} of soil, whether PGPR treatment improved growth, and if remediation could be measured. Annual ryegrass was planted on site 1. To determine the protective effect of PGPR on the plants, treatments were plants with and without PGPR. In 2004, the weather conditions were optimal, and excellent growth of PGPR-treated annual ryegrass was achieved with more than a 40% increase in shoot length and a 2–3-fold increase in ground cover compared to untreated plants (Table 1). Dispersal of PGPR throughout the site, due to tilling, could not be controlled or determined in the spring of year 2; therefore, to avoid experimental artifacts, only PGPR-treated plants were planted on site 1 in years 2 and 3. Concurrently, starting in year 2 (2005), a series of small plots in a paired block design were set up on site 2, to examine plant growth and remediation with PGPR-treated and untreated plants.

TABLE 1. (A) Plant Shoots Measurements (mm) of Annual Ryegrass in Year 1 at Site 1 ($n=10$, ±SD), and (B) Ground Cover Measurements in Years 1 and 2 at Experimental Sites 1 and 2 ($n = 3$, ±SD)[a]

date	site	plant type	− PGPR	+ PGPR
			shoot length (mm)	
07/2004	1	AR	91.8 ± 22	128.7 ± 35[b]
08/2004	1	AR	191.0 ± 28	274.9 ± 32[b]
			ground cover (%)	
07/2004	1	AR	23 ± 8	74 ± 9[b]
08/2004	1	AR	38 ± 8	85 ± 4[b]
08/2005	1	AR/TF	N/A	76 ± 9
08/2006	1	AR/TF/B	N/A	87 ± 6
06/2005	2	B/FR	45 ± 5	70 ± 6[b]
06/2005	2	AR/TF	~5	~5
07/2005	2	B/FR	60 ± 4	83 ± 8[b]
07/2005	2	AR/TF	9 ± 6	14 ± 6
08/2005	2	B/FR	5 ± 1	45 ± 7[b]
08/2005	2	AR/TF	~5	~5

[a] Ground cover measurements were performed by estimating vegetation cover in a 1 m^2 area. AR, annual ryegrass; TF, tall fescue; B, barley; FR, fall rye; N/A, not applicable. [b] There are significant differences between control and treated samples with $P < 0.01$.

Biomass accumulation and photosynthetic performance were also determined for plants with and without PGPR on site 2 in years 2 and 3.

Effect of PGPR on Plant Biomass, Plant Length, and Ground Cover. During the course of the study, petroleum contamination was at its highest (~130 g kg^{-1}) in the first year of planting at site 1 (Figure 1). Plants without PGPR grew poorly; plant size and ground cover were much greater for PGPR-treated plants (Table 1). In 2005, similar effects were seen when site 2 was planted for the first time using the same experimental protocols (Table 1). Soil contamination decreased in each successive year, while plant growth improved (Figure 1 and Table 1). Each year, plants with PGPR inoculation germinated and grew well, and exhibited fewer signs of phytotoxicity, such as stunted growth, lower seed yield, and extent of chlorosis, than untreated plants. Plant growth measurements showed a consistent improvement in shoot length (Table 1) and plant biomass (SI Figure S2) when PGPR were used. Greatly increased ground coverage was also evident in plots with PGPR-treated plants, relative to plots with untreated plants (Figure 1 and Table 1). Note that, in 2005 on site 2, the annual ryegrass and tall fescue mixture did not grow well (<5% ground cover) due to early season drought, and this was the only case where PGPR improved plant growth was not observed (Table 1).

Effect of PGPR on Plant Photosynthesis. Small, yet significant, differences in photosynthetic parameters were apparent when different plant species were examined using PAM fluorometry. An increase in qN, the nonphotosynthetic quenching parameter, has been used as an indicator of plant stress. In 2005, qN was lower in both mixtures of plant species (tall fescue and annual ryegrass, fall rye and barley) treated with PGPR than in their untreated counterparts (Figure 2). No significant changes in qP were detected in either 2005 nor 2006. In 2005, PGPR-treated mixture of fall rye and barley plants had higher F_v/F_m and yield values than untreated plants. However, PGPR-treated tall fescue and annual ryegrass mixture showed lower F_v/F_m and yield values than untreated plants. In 2006, PAM fluorometry did not indicate major differences between plants with and without PGPR, aside from a small change in barley treated with PGPR where qN appeared to increase with PGPR treatment. Thus, PAM fluorometry measurements of photosynthesis indicate that both annual ryegrass and tall fescue, and barley and fall rye

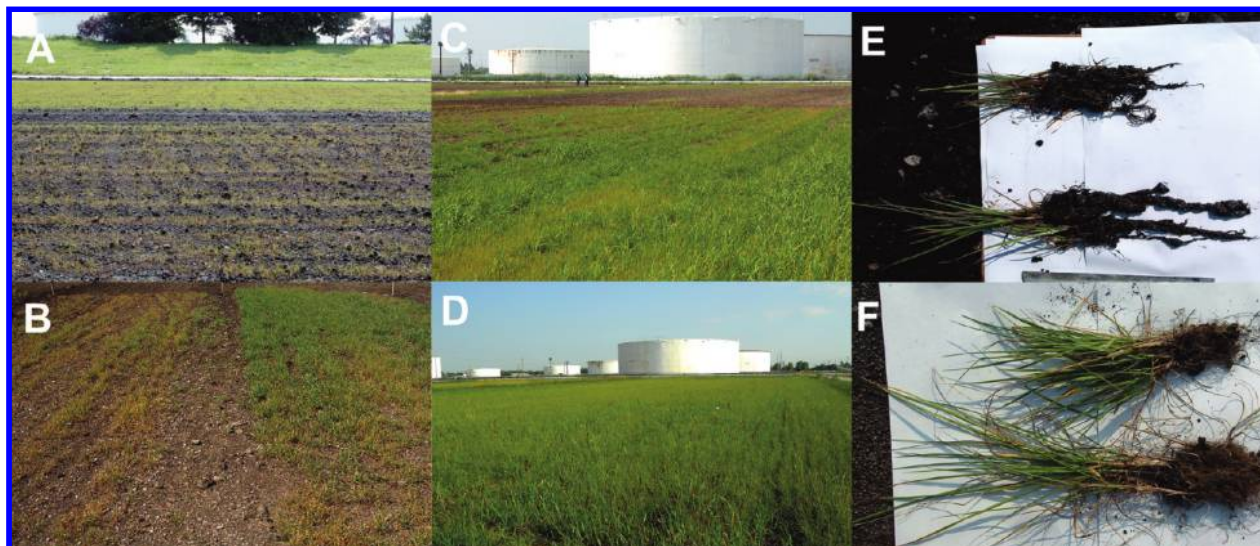

FIGURE 1. Effectiveness of PEP at promoting plant growth in TPH contaminated soils at site 1 in 2004 growing season using annual ryegrass, 60 days after planting (foreground is PGPR, background is + PGPR) (A), site 2 in 2005 using barley and fall rye, 35 days after planting (left is PGPR, right is + PGPR) (B), site 1 in 2005 using tall fescue and annual ryegrass, 35 days after planting (C), and at site 1 in 2006 planted with barley, tall fescue, and annual ryegrass, 110 days after planting (D). Annual ryegrass from site 1 in 2004, 60 days after planting (E), and 120 days after planting (F); top plants are − PGPR, bottom plants are + PGPR plants.

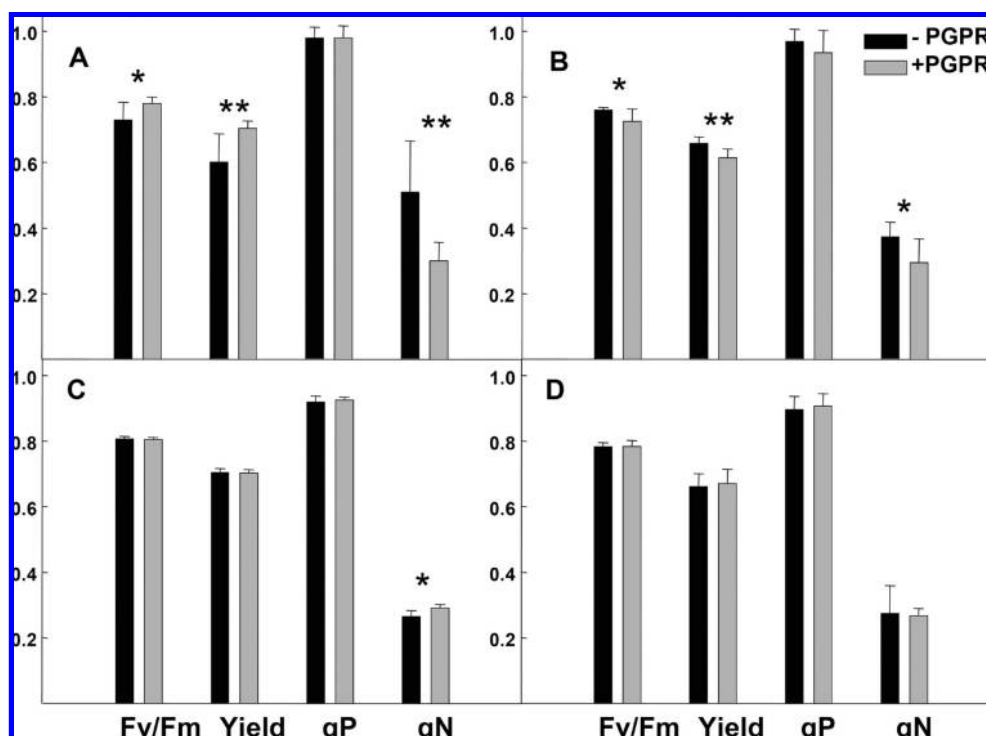

FIGURE 2. PAM parameters obtained at site 2 from barley and fall rye in 2005 (A), annual ryegrass/tall fescue in 2005 (B), barley in 2006 (C), and annual ryegrass/tall fescue in 2006 (D). Measurements are the mean (±SD) from two sampling events ($n = 6$) where similar trends were seen. Asterisks indicate significant differences between PGPR and untreated samples, *$P < 0.05$ and ** $P < 0.01$.

mixtures were performing better with PGPR treatment, according to qN parameter. Further, annual ryegrass and tall fescue treated with PGPR were better able to thrive in a contaminated environment than untreated plants according to F_v/F_m and yield parameters.

Phytoremediation of TPH. In year 1 (2004), annual ryegrass was grown on site 1 with and without PGPR. Plants with PGPR grew very well and approximately 50% TPH remediation was observed (Figure 3). PEP was able to lower the concentration of specific CCME fractions, also on the order of 50% (Figure 3). Without PGPR treatment, plant growth was poor, only approximately 20% remediation was observed and the change in TPH levels relative to the time zero (t_0) control was not significant (Figure 3). In May 2005, PGPR-treated annual ryegrass and tall fescue were planted together on site 1. Plants from the previous year were tilled into the soil prior to sowing. The average TPH concentration at the onset of the 2005 field season was 106.3 g kg^{-1}, greater than the final concentration in 2004 of 65.2 g kg^{-1}. In year 2, significant remediation was once again achieved (Figure 3), total hydrocarbon concentration decreased to 69.6 g kg^{-1} TPH. Most importantly, F3 and F4 were remediated further (Figure 3). In year 3 (2006), following tilling, site 1 was planted with PGPR-treated annual ryegrass, tall fescue and barley.

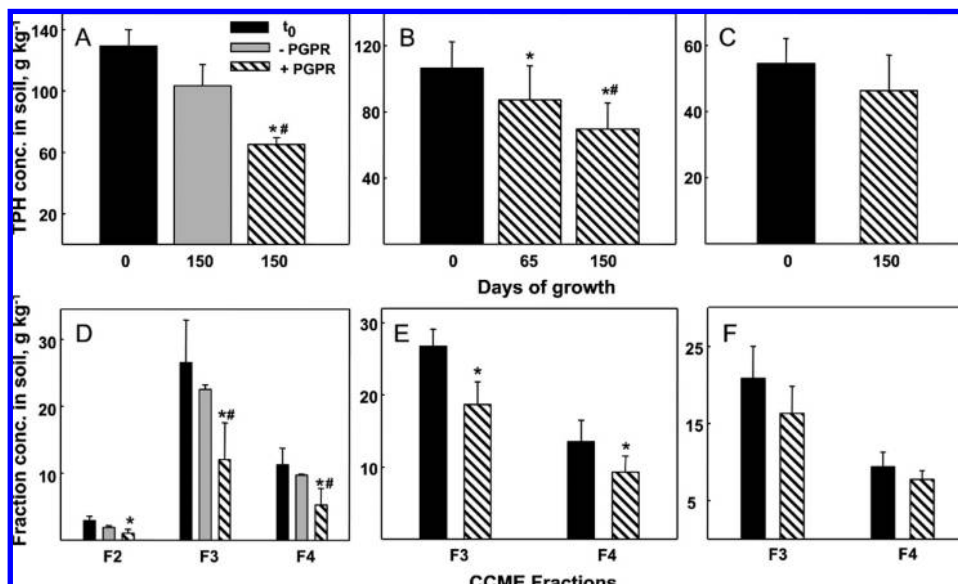

FIGURE 3. Decrease in TPH concentration (A−C), F2, F3, F4 (D), and F3, F4 (E, F) concentrations. TPH concentrations in 2004, using annual ryegrass after a growing period of 150 days ($n \geq 5$) (A), in 2005 using annual ryegrass and tall fescue mixture after a growing period of 65 and 150 days ($n \geq 5$) (B), and in 2006 using annual ryegrass, tall fescue and barley mixture after a growing period of 150 days ($n \geq 5$) (C). CCME fractions F2, F3, and F4 in 2004, at 150 days of growth using annual ryegrass ($n \geq 3$) (D). Fractions F3 and F4 in year 2005 at 150 days of growth using annual ryegrass and tall fescue mixture ($n \geq 3$) (E) and in 2006 using annual ryegrass, tall fescue and barley mixture ($n \geq 3$) (F). Asterisks indicate values significantly different from t_0 control ($P < 0.05$), # indicate values significantly different from 150 days growth, PGPR ($P < 0.1$). In unplanted controls TPH concentration decreased 22%, down to 87.9 ± 5.9 g kg^{-1}. Two replicates were analyzed. Error bars indicate ± SD.

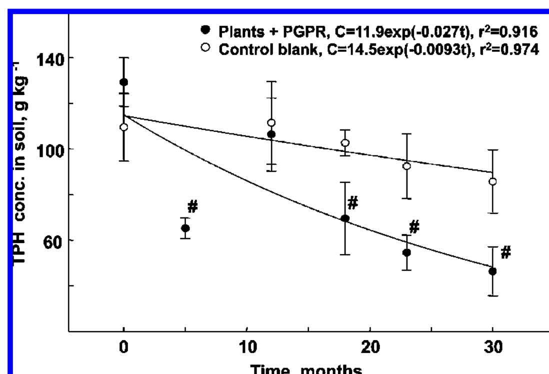

FIGURE 4. Remediation kinetics of PEP and unplanted control. TPH removed from the soil were determined at various time points throughout the 30 month remediation project. Note: blank and planted samples were collected from different parts of the field and thus had slightly different starting concentrations. Error bars indicate ±SD, # indicates values significantly different from time zero from the respective starting concentrations of PGPR planted and unplanted treatments.

Approximately 15% remediation of total hydrocarbons was achieved (Figure 3), with ∼20−25% remediation of both F3 and F4 (Figure 3).

Remediation kinetics for the three year field trial are presented in Figure 4. Overall, 65% remediation was achieved using plants with PGPR, down to 46.4 g kg^{-1}, whereas the unplanted control had a total drop in TPH concentration of only 22%, down to 87.9 g kg^{-1} (note different starting concentration for control and planted areas). While a total drop in TPH concentration in PGPR-treated plants was significant for all but one time point, none of the TPH decreases in unplanted control were significantly different from the zero time point. Data for plants with PGPR and for the unplanted control were fitted to a first order kinetics model. The first order kinetics model was a good fit for the PGPR treatment (r^2 of 0.916) and for the unplanted control (r^2 of 0.974) data. Remediation kinetics remained first order throughout the three years of field trials and the rate constant k for remediation with PGPR-treated plants was 0.027 month^{-1} versus a k of 0.009 month^{-1} without plants.

Remediation was also monitored at site 2 using a series of small plots that were planted for two consecutive years. Remediation data collected (presented in the Supporting Information) showed comparable magnitude of remediation to site 1 and were typically 10−30% each year (SI Table S2). Four times out of five, the PGPR-treated plants had greater levels of remediation than the untreated plants. To create a robust data set for statistical analysis of the effect of PGPR, we pooled the data from all plots into with PGPR and without PGPR (regardless of plant type), thus combining all plant species from both 2005 and 2006. In that case, we were able to show that PGPR-treated plants in small plots had a statistically significant level of remediation of 18% ($p = 0.05$), whereas with the untreated plants remediation was not statistically significant (15% remediation).

Discussion

Successful remediation of TPH in the field has been hindered by low biomass accumulation, particularly when petroleum hydrocarbon levels are high. PGPR have previously been used to improve plant growth in agriculture (*34*) and in greenhouse phytoremediation applications (*19*, *20*). However, few full scale studies have been performed to evaluate PEP in the field (*24*). Our study showed that PEP can be effective, even in highly contaminated soils. Here, we have shown that PEP was more successful relative to natural attenuation or plant growth alone.

The ability of PEP to overcome the limitations of other biological remediation strategies stemmed from its capacity to generate high levels of biomass. PGPR plants are better able to withstand the stress of growing in the contaminated soils than plants without PGPR. This was observed as both the 40% increase in shoot length in year 1 (Table 1), and the 40% increase in shoot fresh weight in PGPR plants in subsequent years (SI Figure S2). Treatment of seeds with PGPR enabled the plants to initiate and sustain more vigorous

growth. This was also evident in the ground cover data which was much greater with PGPR-treated plants compared to untreated plants (Table 1). Addition of PGPR also stimulated root growth, albeit to a smaller degree than shoot growth, putatively resulting in a greater rhizosphere that is likely responsible for remediation of recalcitrant F3 and F4 (*10, 35*). The increase in size of the roots and shoots also would effectively lower the ratio of phytotoxic contaminants to the amount of plant tissue, lowering stress on the plants.

Often, photosynthetic activity can be affected in plants under persistent hydrocarbon stress, such as that coming from polycyclic aromatic hydrocarbons (PAHs), photomodified PAHs, and creosote (*32, 36, 37*). For this reason, photosynthesis was used as an end point. Despite poor growth resulting from the high contaminant load in experimental soils, the plants appeared healthy and expectedly there was not a large degree of photosynthetic stress. Plants without PGPR were able to carry out photosynthesis efficiently with only minor signs of environmental injury. Nonetheless, in 2005, the small negative effects of petroleum contamination that were observed were ameliorated in the presence of PGPR. This was seen as higher F_v/F_m and yield for treated barley and fall rye plants. These data indicate less damage to PSII (*32*). However, in some cases, treated plants had slightly lower yield and F_v/F_m values. In 2005, PGPR-treated annual ryegrass and tall fescue as well as fall rye and barley had lower nonphotosynthetic quenching (qN), indicating less environmental injury. Taken together, treated and untreated plants showed relatively good values for the photosynthetic parameters, confirming our visual observations that the plant tissue produced was healthy.

In 2006, the negative effects of petroleum contamination on growth were not as pronounced, and consistent with this, photosynthesis was even less impacted. The PAM measurements for tall fescue and annual ryegrass in 2006, showed no significant differences between plants with and without PGPR. This could be due to diminished stress as result of lowered phytotoxicity due to remediation in the previous year. Overall, the photosynthetic data suggest that although PGPR may improve photosynthesis, the changes were small and as a result did not contribute to the growth improvement due to PGPR treatment. Thus, improved plant performance is likely due to other factors, such as lowering stress ethylene levels. Through the use of ACC deaminase, PGPR consume the precursor to ethylene, ACC, resulting in improved plant growth, despite the high levels of contaminants present in the soil. In fact, visual observations of the plants did not reveal stress effects, which is consistent with only minor impacts on photosynthesis. This indicates there may be little stress on the plants, so there was no physiological reason for growth to be impeded. Thus, when PGPR is employed, the putative ethylene block on root growth is alleviated allowing more vigorous plant growth without negative impacts on the plants.

It is clear from our findings that favorable conditions for petroleum degradation were created at a highly contaminated site using proper plant selection, land farming techniques and application of PGPR. Phytoremediation was successfully applied to remediate TPH contaminated soils using annual ryegrass, tall fescue, barley, and fall rye. We have shown that PEP continues to remediate soils, with first order kinetics when employed in successive field seasons. During this 30 month trial TPH concentration in the soil declined from 129.3 to 46.4 g kg^{-1}.

In 2004, the weather was excellent for plant growth (moderate temperatures and sufficient precipitation) in the Sarnia, Ontario, region. That year, we observed the most pronounced effects of PEP, both in terms of plant growth and TPH remediation (Figure 1, Figure 3). The increased growth of PGPR plants was reflected in remediation, with approximately 50% decrease in TPH using PGPR-treated plants. Remarkably, both recalcitrant F3 and F4 were remediated. Without PGPR, plants grew poorly and the 20% remediation observed was not significantly different from the control. Without plants no drop in TPH was observed that year. The final TPH concentration at the end of year 1 was 65.2 g kg^{-1}. In 2005, the TPH concentration at the beginning of the 2005 field season rose to 106.3 g kg^{-1}. This was likely due to tilling performed prior to planting. We propose that because root density decreases with increasing soil depth, tilling would bring contaminants from the zone of lower root density, which had experienced less remediation, to the surface. The final concentration of hydrocarbons in the soil at the end of year 2 was found to be 69.6 g kg^{-1}. Thus, 35% remediation was realized in year two. The unplanted control showed a small 8% drop in TPH concentration. In the third year, the initial TPH concentration in April 2006 was 54.6 g kg^{-1} TPH. In contrast to the previous year, the TPH concentration dropped over the fall and winter, possibly due to plant and/or microbial activity during the fall of 2005 (after our last sampling). Because the plants were not dug into the soil at the end of 2005, remediation continued after final sampling. In 2006, climatic conditions were conducive to phytoremediation, with moderate temperatures and sufficient rainfall. Excellent plant growth was observed as a direct result of lower phytotoxicity, which can be attributed to remediation during the preceding years. In this final year, approximately 15% remediation was achieved for plots planted with PGPR plants, while the unplanted control showed a drop of approximately 7%.

PGPR effects from site 1 in 2004 were further confirmed on site 2 in years 2 and 3, with a series of small plots and a total of five independent trials (SI Table S2). Changes were smaller than on the larger plots, average remediation with PGPR-treated plants of approximately 18% was observed. In four out of five replicates there was small, yet reproducible improvement in remediation with PGPR-treated plants. Reasons for less remediation in smaller plots compared to site 1 may be the possible migration of bacteria from plot to plot, and a larger number of samples being taken from areas of low plant growth due to smaller plot size. Nonetheless, with PGPR treatment the average 18% remediation was statistically significant, whereas in plots without PGPR the remediation was not statistically significant. This mirrored the results from site 1, where only remediation with PGPR-treated plants was statistically significant.

As the TPH levels assessed in this study have high inherent variability in soil samples, cautious interpretation of the above data is necessary, particularly when considering the remediation data for each year independently. Furthermore, technology transfers, such as this one, are often difficult to execute in the field and the resulting remediation kinetics may be unpredictable (*38*). To account for these two points, we examined the site 1 remediation data for the three year trial as a whole and observed a positive correlation between TPH removal and the length of time that PGPR-treated plants were grown, to an extent that could not be described by the analytical and/or sampling variability. The decrease in hydrocarbon concentration in the soil appears to follow first order kinetics over the course of three years (Figure 4), typical of petroleum degradation (*39*). In contrast, unplanted control soils, which did not receive any plant or PGPR treatments, showed only small decreases in TPH levels (Figures 3 and 4) and the changes were not significant. Nonlinear regressions of the decrease in TPH concentration using an exponential decay model (eq 1) showed a trend, with consistency between each year's data cluster and the overall kinetic analysis. In (eq 1), the parameter obtained through this analysis, k, is the first order rate constant for TPH remediation. Combining

the temporal data set throughout the 30 month trial period, provided high statistical significance to this rate constant. Thus, what we obtained was a very robust data set showing consistent decreases in contamination levels over the three year period. With PGPR-treated plants, k was 0.027 month^{-1} versus a k of 0.009 month^{-1} without plants. This clearly shows that it is phytoremediation that is causing the loss of TPH from the soil.

Phytoremediation is a promising alternative to other remedial biotechnologies, due to its potential for increasing the amount of plant biomass available for phytodegradation and microbial degradation of soil contaminants. This system still has limitations, however, when high levels of contaminants hinder plant growth and depress microbial populations. Addition of PGPR addresses these problems by increasing the plant phytotoxicity threshold and greatly enhancing biomass accumulation. Root growth is especially necessary for remediation and if high levels of root biomass can be achieved, this, can stimulate both in planta and ex planta degradation of TPH. Plants increase the degradation rate, particularly if PGPR treatment is used.

In traditional phytoremediation strategies, high molecular weight petroleum fractions (typically F3 and F4) were often resistant to remediation (*20*). Encouragingly, in this field trial, PEP was successful at remediating TPH, including recalcitrant F3 and F4. Most of the compounds in F3 and F4, such as PAHs, fall outside of the favorable uptake range of 0.5−3 log K_{ow} (*11, 40, 41*). These compounds are most likely degraded in the rhizosphere by bacteria, fungi, and PGPR that are nutritionally supported by plant exudates (*8, 10, 11, 40, 41*). Thus, here we have shown the successful application of a PGPR-assisted phytoremediation technology for remediation of a heavily contaminated site containing recalcitrant petroleum compounds. Further, we believe the phytoremediation system can be applied broadly at petroleum impacted sites where other methods have failed.

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Note Added after ASAP Publication

The Supporting Information for this article published on May 15, 2009 contained an error. The correct version published ASAP on May 19, 2009.

Supporting Information Available

Selected chemical and physical soil parameters, including TPH fractions, are listed. Map of the study site is included to describe experimental design over the course of the trial. Remediation data for site 2 in 2005 and 2006 is provided. Plant biomass data for PGPR-treated and untreated plants is provided. Comparison of gravimetric analysis from our laboratory and certified analytical laboratories is provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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